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# Characterization of Vaccinia virus alternative antigen transport and processing pathways for their presentation to cytotoxic $CD8^+$ T lymphocytes

**Tesis Doctoral**

**DAVID GAMARRA CARRASCO**

Licenciado en Biología

**Centro de Biología Molecular Severo Ochoa**

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Esta memoria ha sido presentada para optar al grado de Doctor en Biociencias Moleculares (Departamento de Biología Molecular, Facultad de Ciencias) en la Universidad Autónoma de Madrid por el licenciado en Biología:

**David Gamarra Carrasco.**

La Directora de tesis,

**Margarita del Val Latorre,**

Doctora en Ciencias (Químicas – Bioquímica) por la Universidad Autónoma de Madrid. Investigadora Científica del Centro de Biología Molecular Severo Ochoa (CSIC-UAM). Responsable del laboratorio de Inmunología Viral del departamento de Biología Celular e Inmunología,

certifica que esta tesis ha sido realizada bajo su dirección en el Centro de Biología Molecular Severo Ochoa.

Fdo:

Directora de tesis,
Margarita del Val

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## Resumen

Los linfocitos T citotóxicos $CD8^+$ (CTL) exploran la superficie de las células del organismo en busca de anomalías, lo que permite su correcta eliminación. Para que esto sea posible, las células del organismo procesan proteínas tanto endógenas como derivadas de patógenos, generando péptidos que se cargan y presentan en moléculas del complejo principal de histocompatibilidad de clase I (MHC-I). La presentación de antígenos por la vía clásica involucra al transportador asociado a presentación antigénica (TAP). Este transportador permite a los antígenos peptídicos acceder al retículo endoplasmático, donde se asocian los complejos MHC-I. Sin embargo, se han descrito diferentes epítopos que se presentan de forma independiente de este transportador.

Nos propusimos caracterizar la o las proteasas implicadas en la presentación independiente de TAP. Utilizando linfocitos T $CD8^+$ purificados de bazos de ratones C57BL/6 infectados con el virus vaccinia y células dendríticas derivadas de médula ósea (BMDC), estudiamos la importancia del proteasoma en la presentación de antígenos independientes de TAP. Midiendo la activación mediante la técnica de tinción intracelular de citoquinas (ICS) de los linfocitos T $CD8^+$ purificados estimamos que tras el tratamiento con el inhibidor del proteasoma lactacistina (LC) a BMDC de ratones C57BL/6 y $TAP1^{-/-}$, la inhibición de la presentación de antígeno es fuerte y similar en ambos casos. Por tanto, al igual que en las BMDC silvestres, el proteasoma juega un papel principal en la presentación de antígenos en BMDC deficientes en TAP.

Con el objetivo de caracterizar proteasas alternativas involucradas en el procesamiento de antígenos virales estudiamos, para una batería de ocho epítopos, la activación de CTL monoespecíficos tras estimularlos con BMDC infectadas y tratadas con inhibidores de diferentes proteasas. Sólo observamos un efecto sobre la presentación antigénica tras el tratamiento con el inhibidor de pro-protein convertasas dec-RVKR-cmk, que inhibió en gran medida la presentación de uno de los ocho epítopos.

Una vez conocida la existencia de mecanismos alternativos de presentación en MHC-I, quisimos saber si estos tenían relevancia fisiológica en una infección por el virus vaccinia. Para ello, inoculamos tanto ratones silvestres como $TAP1^{-/-}$ con BMDC cargadas con péptidos sintéticos. Tras catorce días, infectamos los ratones con virus vaccinia y medimos la carga viral a diferentes días post-infección y en diferentes órganos. En bazo, observamos un efecto protector de los tres péptidos tanto en los ratones silvestres como en los ratones $TAP1^{-/-}$. Estos resultados sugieren que distintos tipos celulares podrían tener activas distintas vías alternativas de procesamiento antigénico, siendo más relevantes en unos órganos que en otros. También, confirman la existencia de las vías independientes de TAP descritas previamente en el laboratorio para los epítopos estudiados.

## Abstract

CD8+ cytotoxic T lymphocytes (CTL) screen the surface of the cells in the organism in seek of abnormalities, which allows their correct elimination. This is possible because the cells in the organism process proteins, either self-proteins or pathogen-derived. This degradation generates peptides that are loaded and presented on class I major histocompatibility complexes molecules (MHC-I). The presentation of antigens through the classical pathway involves the transporter associated to antigen processing (TAP). This transporter allows peptidic antigens access the endoplasmic reticulum, where they are loaded onto MHC-I complexes. However, different epitopes that are presented in a TAP-independent manner have been described.

We aimed to characterize the protease or proteases involved in the TAP-independent antigen presentation. Using $CD8^+$ T lymphocytes purified from spleens from VACV-infected C57BL/6 mice and dendritic cells derived from bone marrow (BMDC), we studied the importance of the proteasome in TAP-independent VACV antigen presentation. Measuring $CD8^+$ T lymphocyte activation by intracellular cytokine staining (ICS), we estimated that, after treatment of C57BL/6 and $TAP1^{-/-}$ VACV-infected BMDC with the proteasome inhibitor lactacystin (LC), the inhibition of the antigen presentation is strong and similar in both cases. Therefore, just like in wild type BMDC, proteasomes play a key role in TAP-independent VACV antigen presentation in BMDC.

With the aim of characterizing alternative proteases involved in the antigen processing we studied, for eight peptides, the activation of monospecific CTL after VACV-infected BMDC stimulation. These BMDC were also treated with inhibitors of different proteases. We only observed an effect on antigen presentation after the treatment with the pro-protein convertases inhibitor dec-RVKR-cmk, which inhibited the presentation of one epitope.

Once we acknowledged the existence of alternative MHC-I antigen presentation mechanisms, we wanted to know whether these had any physiological relevance on a VACV infection. For that, we inoculated C57BL/6 as well as $TAP1^{-/-}$ mice with synthetic peptide-loaded BMDC. After fourteen days, we infected the mice with VACV and measured the viral load at different days post-infection and in different organs. In spleens we observed a protective effect by the three peptides included in the study in C57BL/6 as well as in $TAP1^{-/-}$ mice. These results suggest that different cell types may have different alternative antigen processing pathways activated, being more relevant in some organs than in others. Also, they confirm the existence of previously described VACV antigen processing TAP-independent routes.

“Quien no sabe lo que busca no entiende lo que encuentra”

Claude Bernard (1813-1878)

## INDEX

## 1. ABBREVIATIONS.

| | |
|---|---|
| aa | amino acid |
| ABC | ATP-binding cassette |
| ACE | Angiotensin convertase enzyme |
| APC | Antigen presenting cell |
| AraC | Cytosine arabinoside |
| BFA | Brefeldin A |
| BH | Bleomycin hydrolase |
| BHV | Bovine herpes virus |
| BMDC | Bone marrow-derived dendritic cells |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| CMV | Murine cytomegalovirus |
| COP | Copenhagen |
| CPRG | Chlorophenol red-β-D-galactopyranoside |
| CPXV | Cowpox virus |
| CTL | CD8+ Cytotoxic T Lymphocytes |
| Cyt | Cytoplasmic |
| d.p.i. | Days post-infection |
| DC | Dendritic cell |
| dec-RVKR-cmk | Decanoyl-Arg-Val-Lys-Arg-chloromethylketone |
| DNA | Deoxyribonucleic acid |
| DPP-III | Dipeptidyl-peptidase III |
| DRiPs | Defective ribosomal products |
| E:T | Effector:Target |
| EBV | Epstein Barr Virus |
| EHV | Equine herpes virus |
| Epo | Epoxomicin |
| ER | Endoplasmic reticulum |
| ERAD | Endoplasmic-reticulum-associated degradation |
| ERAP | Endoplasmic reticulum aminopeptidase |
| EV | Extracellular virion |
| FBS | Fetal bovine serum |
| GFP | Green fluorescent protein |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| HCMV | Human cytomegalovirus |
| HIV | Human immunodeficiency virus |
| HSV | Herpes simplex virus |
| ICS | Intracellular cytokine staining |
| IDE | Insulin-degrading enzyme |
| IFN | Interferon |
| IL | Interleukin |
| IRAP | Insulin-responsive aminopeptidase |
| IV | Immature virion |
| LacZ | Bacterial beta-galactosidase gene |

| | |
|---|---|
| LAP | Leucine aminopeptidase |
| LC | Lactacystin |
| $LC^E$ | LC-enhanced |
| $LC^R$ | LC-resistant |
| $LC^S$ | LC-sensitive |
| LPS | Lipopolysaccharide |
| LV | Lentivirus |
| m.o.i. | Multiplicity of infection |
| MALDI-TOF | Matrix-assisted laser desorption/ionization time-of-flight |
| MHC | Major histocompatibility complex |
| MHC-I | Major histocompatibility complex class I |
| mRNA | messenger ribonucleic acid |
| MV | Mature virion |
| NBD | Nucleotide-binding domain |
| NK | Natural killer |
| NLVS | Tri-leucine vinyl sulfone |
| NP | Nucleoprotein |
| OHM | Optimal matching hydrophobicities |
| ORF | Open reading frame |
| OVA | Ovalbumin |
| PBS | Phosphate-buffered saline |
| PC7 | Proprotein convertase 7 |
| PEC | Peritoneal exudate cells |
| PFA | Paraformaldehyde |
| PFU | Plaque-forming unit |
| PLC | Peptide loading complex |
| PR | Post-replicative |
| PRV | Pseudorabies virus |
| PSA | Puromycin-sensitive aminopeptidase |
| r.p.m. | Revolutions per minute |
| R.T. | Room temperature |
| RNA | Ribonucleic acid |
| RQ | Relative quantification |
| RT-qPCR | Reverse transciption quantitative polymerase chain reaction |
| rVACV | Recombinant vaccinia virus |
| rVACV-OVA | Recombinant VACV expressing full-length OVA |
| rVV-GFP | Recombinant VACV expressing GFP |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEM | Standard error of the mean |
| SNARE | Soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor |
| SP | Signal peptidase |
| SPP | Signal peptide peptidase |
| SV40 | Simian virus 40 |

| | |
|---|---|
| TAP | Transporter associated to antigen processing |
| TAP-dep | TAP-dependent |
| TAP-ind | TAP-independent |
| TAPL | Transporter associated to antigen processing-like |
| TCR | T Cell Receptor |
| TEIPP | T-cell epitopes associated with impaired peptide processing |
| TM | Transmembrane |
| TMD | Transmembrane domain |
| TOP | Thimet oligopeptidase |
| TPPII | Tripeptidyl-peptidase II |
| VACV | Vaccinia virus |
| WR | Western reserve |
| WT | Wild type |
| WV | Wrapped virion |
| z-VAD-fmk | N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone |
| β2m | β2 microglobulin |

# 2. INTRODUCTION.

## 2.1. MHC-I antigen processing.

$CD8^+$ Cytotoxic T Lymphocytes (CTL) screen the surface of all cells in the organism in order to detect tumors or infections caused by a pathogen and thus, eliminate the tumoral or infected cell. Their T Cell Receptor (TCR) recognizes peptides displayed at the plasma membrane by major histocompatibility complex (MHC) class I molecules. These peptides are derived from the proteolytic processing of endogenous proteins either from the host or from the pathogen. In professional antigen presenting cells (APC) these peptides can also derive from the processing of exogenous proteins in a process named cross-presentation. These peptides have an average length of 9 aminoacids (aa) and they are frequently derived from larger precursors that require a proteolytic trimming previous to MHC class I (MHC-I) loading. The specific recognition of MHC/peptide complex by the TCR triggers CTL activation and proliferation, promoting the tumoral or infected cell elimination by their cytotoxic activity or cytokine release.

In the classical antigen processing pathway, antigenic peptides are originated by cytosolic degradation of endogenous proteins or defective ribosomal products (DRiP) via the proteasome. The products generated by the proteasome are mainly peptides shorter than 8 aa, although it also generates peptides of 8 aa or longer that may contain epitopes or their precursors. These precursors might have in their amino termini extensions of several aa that can be trimmed by cytosolic aminopeptidases. Thus, peptides generated in the cytosol are transported to the endoplasmic reticulum (ER) through the transporter associated with antigen processing (TAP). Further trimming of these peptides can be performed by either ER aminopeptidases (ERAP) or the carboxy-dipeptidase angiotensin convertase enzyme (ACE). At this point, peptides are loaded onto a complex formed by nascent heavy MHC-I chains and β2 microglobulin (β2m) with the aid of the chaperones tapasin, calreticulin and ERp57. The complex formed by TAP, tapasin, calreticulin, ERp57 and MHC-I is known as the peptide loading complex (PLC). The stable MHC/peptide complex is further transported through the constitutive secretory pathway to the plasma membrane, where $CD8^+$ cytotoxic T lymphocytes efficiently recognize and eliminate the infected cell.

### 2.1.1. Proteases involved in MHC-I antigen processing.

#### 2.1.1.1. Proteasomes.

The proteasome is a multiproteic complex that functions as the main degradative endoprotease in the cell. It is highly conserved and is found in cytosol and nucleus. The 26S

proteasome is a multiproteic complex with proteolytic activity that belongs to the ubiquitin-proteasome system. The enzymatic cascade E1, E2 and E3 transfers multiple ubiquitins to ε-amino groups in lysine residues localized in protein targets to be degraded by the proteasome. The 26S proteasome is formed by 33 canonical subunits arranged into two complexes: a 20S catalytic core and two 19S regulatory subunits flanking the core [19S-20S-19S] (Wehmer and Sakata, 2016). These regulatory subunits are responsible for the binding and unfolding of polyubiquitylated substrates. The 20S core is constituted by 28 different subunits and are organized forming 4 rings that stack together to shape a barrel-like conformation (Groll *et al.*, 1997). Both external rings are constituted by 7 α subunits (α1-α7) that avoid the recruitment of non-selective substrates. The interaction between 20S core, the activating complex PA28 and the regulative 19S subunit produces a change in the orientation of external α subunits that allow the entry of substrates and the release of products. Each of the 2 inner rings is constituted by 7 catalytic β subunits (β1-β7) that form an inner pocket where substrate degradation can take place. The catalytic subunits in the active site have different proteolytic activities; caspase-like (β1), trypsin-like (β2) and chymotrypsin-like (β5). These activities vary among species, cell types and disease state (Priestman *et al.*, 2014).Proteasome cleavage specificity is low since any aa can serve as a cleavage site.

There are several reasons why the proteasome has been implicated in the generation of MHC-I epitopes:

First, specific inhibitors of the proteasomal activity suppress almost completely the presentation of some MHC-I epitopes. Proteasomes also strongly affect, although to different extents for different allotypes, MHC-I molecule expression at the cell surface. Also, the lack of carboxypeptidase activity in the cytosol (Reits *et al.*, 2004; Reits *et al.*, 2003) and in the ER of rat cells (Powis *et al.*, 1996) showed that the C-termini of MHC-I ligands must be generated by an endoprotease. In fact, it has been demonstrated that the proteasome generates the exact carboxy termini of some epitopes presented to CTL (Shastri *et al.*, 2005). However, it has been recently described an important role for the carboxy-dipeptidase angiotensin convertase enzyme (ACE) in the generation of the carboxy termini of some epitopes in the secretory pathway (Shen *et al.*, 2011). This finding has questioned the proteasome as the sole MHC-I ligand carboxy terminus generating enzyme.

Secondly, the proteasome generates peptides from 3 to 22 residues. Fifteen percent of them have the optimal length to bind MHC-I molecules (8-10 aa long). On the other hand, 20% of the peptides generated by the proteasome are longer than 8-10 aa, being potential precursors of the final peptide (Kisselev *et al.*, 1999).

Finally, interferon (IFN) γ induces the incorporation of new β-catalytic subunits to nascent proteasomes, LMP2, MELC-1 and LMP7, and they replace the constitutive catalytic subunits β1, β2 and β5, respectively. The resulting proteasome is denominated immunoproteasome, and it is constitutively localized in lymphoid tissues such as thymus, spleen or lymph nodes and in other cell types under IFNγ stimulation. Immunoproteasomes do not have the same cleavage specificity as standard proteasomes, which could change the repertoire of peptides generating a different peptide pool or generating the same peptides with different efficiencies (Kloetzel and Ossendorp, 2004; Sijts and Kloetzel, 2011; Sijts and Kloetzel, 2011).

The main role of the proteasome is the degradation of unfolded proteins for aa recycling or the turnover of proteins. Proteins that get into the ER and fail to pass the quality control requirements must be retrotranslocated to the cytosol for their degradation in the endoplasmic-reticulum-associated degradation (ERAD) pathway, involving the Sec61 channel also known as translocon (Wiertz *et al.*, 1996). DRiP represent the main, but not the sole, source of peptides that are presented by MHC-I. The DRiP hypothesis explains how fast viral peptides are presented on the infected cell surface, since it links the defective protein translation to the antigen processing machinery (Del Val and López, 2002; Princiotta *et al.*, 2003; Yewdell and Nicchitta, 2006; Dolan *et al.*, 2011).

Under proinflammatory conditions, a big amount of unfolded and oxidized proteins is generated, exceeding the degradative activity of the ubiquitin-proteasome system. As a result, these proteins are accumulated in the cell, unbalancing the protein turnover. The main role of the immunoproteasome in these conditions is to maintain the protein homeostasis. Also, the combination of both activities, proteasomal and immunoproteasomal, leads to an enrichment of the generation of peptides available for antigen presentation by MHC-I.

Even though the inhibition of the proteasome has indeed a deep effect in MHC-I ligands generation (Kessler *et al.*, 2003), for certain epitopes proteasomal cleavage can be necessary but not sufficient, since further trimming of proteasome-generated precursors by other proteases might be needed (Kessler *et al.*, 2011; Shen *et al.*, 2011). Thus, the sensitivity to proteasome inhibitors in the presentation of an epitope does not exclude the participation of other proteases in its generation.

#### 2.1.1.2. Cytosolic aminopeptidases and endopeptidases.

Antigen processing involves other proteases apart from the proteasome. They are individually described and summarized in **Table 1**.

The cytosol is a highly degradative environment, and it is the location of many peptidases that rapidly degrade peptides. Particularly, the analysis of living cells incubated with labeled peptides, shows that these peptides are degraded within seconds by aminopeptidases, since N-terminally-protected peptides are stable(Reits *et al.*, 2004; Reits *et al.*, 2003). Cytosolic aminopeptidases cooperate to degrade peptides into aa, a key process for cell survival. They can also trim the amino terminus of extended precursors, resulting either in the generation of the correct MHC-I ligand terminus, or in its destruction. Although most proteasomal products require further proteolytic trimming in order to generate the correct amino terminus, it has been described an unusual natural ligand that is 15 aa long with 6 extended aa in its amino terminus that are not trimmed and is correctly presented to CTL (Samino *et al.*, 2006).

Several cytosolic aminopeptidases have been described, such as the leucine aminopeptidase (LAP), bleomycin hydrolase (BH) or the puromycin-sensitive aminopeptidase (PSA). LAP is a metallopeptidase with preference for hydrophobic aa in the amino terminus (Turzynski and Mentlein, 1990). It is induced by IFNγ and promotes the *in vitro* generation of an epitope from an N-terminally-extended peptide (Beninga *et al.*, 1998). PSA and BH can trim N-terminal extensions from long peptides to generate *in vitro* the correct epitope (Lévy *et al.*, 2002; Stoltze *et al.*, 2000). However, there are epitopes that do not require these aminopeptidases at all. Mice deficient in each of these peptidases show a normal surface MHC-I expression and they exhibit the same $CD8^+$ T lymphocyte response against viral epitopes as wild-type mice (Towne *et al.*, 2005; Towne *et al.*, 2007). Only in splenic DCs deficient in PSA, an increase in MHC-I expression has been observed, but it does not correlate with a better antiviral $CD8^+$ T lymphocytic response (Towne *et al.*, 2008).

| **Subcellular localization** | **Non-proteasomal proteolytic enzymes** | |
|---|---|---|
| | **Collaborative** | **Alternative** |
| **Cytosol** | Leucine aminopeptidase (LAP) | Tripeptidyl-peptidase II (TPPII) |
| | Bleomycin hydrolase (BH) | Caspases 3, 5, 8 and 10 |
| | Puromycin-sensitive aminopeptidase (PSA) | Insuling-degrading enzyme (IDE) |
| | Thimet oligopeptidase (TOP) | |
| | Nardilysin | |
| | Dipeptidyl-peptidase III (DPP-III) | |
| **ER and vesicular pathways** | ERAP1 and ERAP2 | Signal peptidase (SP) |
| | | Signal peptide peptidase (SPP) |
| | | Furin |
| | Proprotein convertase 7 (PC7)* | Cathepsins |
| | Angiotensin convertase enzyme (ACE) | |
| | Insuyline responsive enzyme (IRAP) | |
| | **Transport mechanisms** | |

| | |
|---|---|
| **ER** | TAP |
| | Signal sequence via Sec61 |

**Table 1. Non-proteasomal proteolytic enzymes and transport mechanisms involved in MHC-I antigen processing.**

Different proteolytic activities and transport mechanisms involved in collaborative with proteasome or completely proteasome-independent (alternative) pathways, as well as their subcellular localization, are summarized and compared. *Collaboration with proteasome is unknown.

Tripeptidyl-peptidase II (TPPII) is a cytosolic aminopeptidase that belongs to the subtilisin family and participates in many cellular processes, such as cell division, apoptosis or diseases like obesity and cancer. TPPII removes tripeptides from substrates in their amino termini unless it finds a proline (Tomkinson, 1999). It has also trypsin-like endoprotease activity. Peptides longer than 15 aa are processed mainly by TPPII rather than other aminopeptidases, so TPPII could play an important role as a cytosolic peptidase of large proteasomal products (Reits *et al.*, 2004; York *et al.*, 2006). TPPII was once proposed as a substitute of the proteasome when the latter is inhibited, since cells adapted to grow in the presence of the proteasome inhibitor tri-leucine vinyl sulfone (NLVS) overexpress the protease TPPII (Glas *et al.*, 1998; Geier *et al.*, 1999). However, as it has been reviewed (van Endert, 2008), antigen presentation by MHC-I was strongly compromised in NLVS-adapted cells (Kessler *et al.*, 2003). Therefore, TPPII is currently understood as a cooperator instead of a substitute of the proteasomal activity (van Endert, 2008).

Several studies describe many endoproteases that either complement the action of the proteasome or act independently, generating epitopes presented by MHC-I.

Caspases are endoproteases that belong to a cytosolic cysteine-proteases family and are involved in the inflammatory response and apoptotic processes. It is known that caspases 5 and 10 participate in a proteasome-independent manner in the production of an epitope of murine cytomegalovirus (CMV) in apoptotic cells infected by a recombinant vaccinia virus (rVACV). Only the inhibition of both proteases together with the proteasome succeeds in completely eliminating epitope presentation (López *et al.*, 2010).

The insulin-degrading enzyme (IDE) is an ubiquous metalloprotease that cleaves insulin chain and peptides smaller than 12 kDa. This enzyme recognizes the substrate conformation and cleaves preferentially substrates with a beta sheet primary structure. There is an epitope of the tumoral protein MAGE-3 presented by HLA-A1 whose processing occurs in a proteasome-independent manner and is cleaved in the cytosol by IDE (Parmentier *et al.*, 2010).

The thimet oligopeptidase (TOP) is a ubiquous cytosolic endoprotease whose main action results in epitope degradation (Saric *et al.*, 2001), though it can also generate precursor peptides (Saric *et al.*, 2004), such as the case of the tumoral protein MART-1 (Kessler *et al.*, 2011). TOP can also trim peptides generating the carboxy terminus of a MHC-I ligand by removing 2 or 3 residues from it. That is the case of an epitope from the tumoral protein PRAME, whose carboxy terminus generation is resistant to proteasome inhibitors (Kessler *et al.*, 2011).

Nardilysine is a cytosolic endopeptidase that recognizes dibasic motifs. It participates in the generation of precursor peptides of the tumoral protein PRAME and in the correct amino terminus of the epitope EBNA3C (Kessler *et al.*, 2011).

Even though testing these cytosolic proteases has been included in several studies with proteasome inhibitors-resistant epitopes, some have failed in finding a cytosolic protease responsible for the generation of a given epitope (Wherry *et al.*, 2006). There is not a unique explanation for this: (1) The inhibition of the proteasome is not complete and the residual activity might be sufficient to generate the epitope (Wherry *et al.*, 2006), (2) the altered pattern of the proteasomal activity caused by its inhibition (Garcia-Medel *et al.*, 2011), might be able to generate the epitope and (3) other proteases, either resident in the secretory pathway, or non-described are responsible for the epitope generation when the proteasome activity is impaired.

#### 2.1.1.3. ER aminopeptidases.

Compared to the cytosol, the ER shows a low proteolytic activity. Apart from the endoproteases signal peptidase (SP) and the signal peptide peptidase (SPP), there are other resident aminopeptidases in the ER that able to remove flanking residues from the antigenic peptide in its amino terminus.

ER aminopeptidases belong to the group of zinc-binding metallopeptidases. This subfamily has 3 members in humans, which are relevant to antigen processing. These are the enzymes ERAP1, ERAP2 and IRAP (insulin-regulated aminopeptidase). In mice, ERAAP acts as an ERAP1 homologous protein, whereas ERAP2 is not expressed. In contrast to IRAP, ERAP enzymes are inducible by IFNγ. Studies that use *in vitro* digestions show that ERAP1 trims optimally 9-to-16 aa-long peptides, and its products are rarely shorter than 8 or 9 aa. This suggests that this ER aminopeptidase has evolved to generate peptides with a proper length for the binding to MHC class I molecules (Serwold *et al.*, 2002; Saric *et al.*, 2002; York *et al.*, 2002). This is consistent with TAP specificity to bind and translocate peptides to the ER, as well as with MHC-I allotypes specificity for peptide binding. About its

specificity, ERAP1 cleaves more efficiently from the precursor peptide big and hydrophobic residues either in the amino or carboxy termini. On the other hand, cleavage of charged or small hydrophobic residues is poorly efficient (Saveanu *et al.*, 2005; Chang *et al.*, 2005; Hearn *et al.*, 2009). However, ERAP1 is not able to cleave peptides with proline in position 2. As a consequence these peptides, which come from larger cytosolic precursors, are accumulated in the ER. This successfully explains the preferential use of proline in position 2 as binding motif of a considerable amount of MHC-I allotypes; whereas TAP is unable to transport these peptides (Serwold *et al.*, 2001). ERAP1 trimming is also determined by inner positions 2, 5 and 7, having preference for hydrophobic or positively charged aa (Evnouchidou *et al.*, 2008).

The resolution of ERAP1 crystallographic structure (Kochan *et al.*, 2011; Nguyen *et al.*, 2011) suggests that this enzyme undergoes conformational changes that would explain the low specificity for its substrate and the preference for large substrates. This structure fits better with the hypothesis of considering ERAP1 as a “molecular rule” (Chang *et al.*, 2005) rather than the alternative “template“ model that proposed that the enzymatic action of ERAP1 occurred on peptides bound to murine MHC class I molecule (Kanaseki *et al.*, 2006). The “template” model has already been refuted by experiments in our laboratory with a 15 aa peptide from human immunodeficiency virus (HIV), a natural precursor that is found in infected cells and is trimmed by ERAP *in vitro* only when it is free from the murine MHC-I H-2L$^{d}$ molecule (Samino *et al.*, 2006; Infantes *et al.*, 2010).

It has been observed that CD8$^{+}$ T lymphocytes repertoire is altered in ERAP-deficient mice, thus indicating that trimming of precursors is already occurring during CD8$^{+}$ T lymphocytes selection in the thymus. However, although in these mice MHC-I molecules expression is diminished, this study failed in finding an effect in the response of CD8$^{+}$ T lymphocytes against different viral antigens (Hammer *et al.*, 2007). Different studies using interference RNA in cell culture have shown the contribution of ERAP1 as much as in the generation as in the destruction of different epitopes for their presentation on MHC-I to CTL (Serwold *et al.*, 2002; York *et al.*, 2002; Zervoudi *et al.*, 2013). There are also evidences that ERAP deficiency compromises the cross-presentation of some particulate antigens (Yan *et al.*, 2006; Firat *et al.*, 2007a).

ERAP2 shows preference for substrates with basic residues either in the amino or carboxy termini. It can form dimeric complexes with ERAP1 increasing the efficiency in cleaving precursors (Saveanu *et al.*, 2005).

The existence of ERAP inhibitors in cytomegalovirus shows the importance of this enzyme in antigen processing (Kim *et al.*, 2011). It has also been possible to identify a CD8$^{+}$

T lymphocyte population that specifically recognizes the FL9 peptide presented by the non-conventional Qa-1b MHC-I molecule, that is only generated when ERAAP activity is impaired (Nagarajan *et al.*, 2012).

Several groups have studied the cleavage proteolytic activity in the cytosol and in the ER. Based on a bioinformatic study, Schatz et al. have analyzed the frequency of aa between positions N5 and N1, being N5 the initial most amino terminal residue of the amino-extended peptide, of the precursors of more than 1500 known epitopes and they have identified the existence of an “amino-terminal processing motif”. Amino-extended precursors have aa like proline or tryptophan in the positions N4-N5. These precursors are previously trimmed in the cytosol and gain access to the ER with extensions of 1 to 3 aa like leucine or methionine or basic aa that would be removed by ER aminopeptidases. The relaxed cleavage specificities of cytosolic and ER aminopeptidases would fit with this amino-terminal degradation motif (Schatz *et al.*, 2008).

#### 2.1.1.4. Carboxypeptidases in the secretory pathway.

Until 2011, the study of the carboxypeptidase activity in the secretory pathway was based on data in rat cells (Powis *et al.*, 1996), and it was assumed to bevery inefficient (Snyder *et al.*, 1998) . It had also been demonstrated in murine and human cells using recombinant VACV harboring SIINFEKL extended or not in its carboxy terminus that the efficiency in epitope generation was limited 1,000-fold when SIINFEKL was C-terminally extended (Medina *et al.*, 2009).

However, there were clear evidences that in murine and human TAP-deficient cells some carboxy-trimming occurred in natural peptides, either coming from signal sequences (Henderson *et al.*, 1992; Wei and Cresswell, 1992) or from entire proteins (Gil-Torregrosa *et al.*, 1998). Finally, it has been recently described that ACE can generate the carboxy terminus of epitopes from peptides pre-processed by the proteasome. ACE-deficient mice show a new repertoire of unique epitopes, since some arise and some fade (Shen *et al.*, 2011). Indeed, the alteration of MHC-I-restricted peptide repertoire in the absence of ACE is sufficient to promote an immune response when wild type (WT) mice are inoculated with ACE-deficient cells (Gonzalez-Villalobos *et al.*, 2013). In fact, the same authors also reported a protective effect in mice overexpressing ACE after immunization with a Polyoma virus epitope.

Taken together, these studies show an emerging and significant role of ACE in the generation of MHC-I-restricted epitopes.

#### 2.1.1.5. Vesicular endoproteases.

Unlike the cytosol or lysosomes, the secretory pathway and vesicular compartments are poorly degradative. However, different endoproteases that participate in MHC-I antigen processing have been described (**Table 1**).

The signal peptidase (SP) is an enzyme that generates the mature form of secretory and some membrane proteins by recognizing and removing the signal sequence from proteins that translocate co-translationally into the ER. This is a pattern sequence that starts in the amino terminus with 1 to 5 basic residues, has a hydrophobic core with 7 to 15 aa followed by 3 to 7 polar aa and ends up with small and non-polar residues in the carboxy terminus. Cleaved signal sequences are short peptides that need little further trimming to bind MHC-I, but some examples that are trimmed on amino, carboxy or both termini have been described (Weinzierl *et al.*, 2008; Suri *et al.*, 2006; Oliveira *et al.*, 2009). Several examples of TAP-independent epitopes generated by this enzyme have been described (Henderson *et al.*, 1992; Wei and Cresswell, 1992; Bell *et al.*, 2009), although there are also some epitopes that are destroyed (Schlosser *et al.*, 2007).

Thanks to large-scale studies on the MHC-I ligand repertoire employing immunoproteomic and mass spectrometry techniques with non-infected cells, it is known that the signal sequence is the main source of TAP-independent ligands presented by HLA-A2 and HLA-B51. The fact that HLA-A2 easily fits alanine in the carboxy terminal position explains that the signal peptidase can generate the exact carboxy terminus in most of the epitopes presented by this molecule (Weinzierl *et al.*, 2008). In other MHC-I allotypes such as $K^d$, HLA-B51 or Qa-1b, further cleavage of the epitope is needed, either in the amino or in the carboxy termini (Weinzierl *et al.*, 2008; Suri *et al.*, 2006; Oliveira *et al.*, 2009), thus implicating aminopeptidases like ERAP and carboxypeptidases like, maybe, ACE.

The signal peptide peptidase (SPP) is other endoprotease resident in the ER that cleaves in transmembrane domains and inside some signal peptides. It is involved in the processing and presentation of a proteasome and TAP-dependent (TAP-dep) epitope (Bland *et al.*, 2003).

Two proprotein convertases located in the trans-Golgi network, furin and proprotein convertase 7 (PC7), have been found to be involved in the processing in the secretory pathway of some epitopes.

Furin is a proprotein convertase resident in the trans-Golgi network that mediates the maturation of many proproteins like peptidic hormones or viral glycoproteins. This enzyme cleaves preferentially after polybasic residues, having specificity for a RXR/KR motif. The

specific inhibitor dec-RVKR-cmk mimics the 4 aa conforming the binding motif to the protease. The inhibition of this protease with dec-RVKR-cmk blocks the presentation of the epitope 9pp89, generated from the viral construction rVACV sC-A9A to CTL by T2/$L^d$ TAP-deficient infected cells in a dose-dependent manner (Gil-Torregrosa *et al.*, 1998; Gil-Torregrosa *et al.*, 2000).

As described in studies using DC, furin can generate around one third of all $K^b$/SIINFEKL complexes in $TAP^+$ cells, showing that this protease takes part in antigen global presentation in the presence of TAP. Interestingly, furin generates $K^b$/SIINFEKL complexes in $TAP^-$DC five times more efficiently than in $TAP^+$DC. It has also been described that furin generates epitopes recognized by $CD8^+$ T lymphocytes in TAP-deficient mice (Medina *et al.*, 2009).

PC7 is a serin-protease that shows a broad tissue distribution and is localized in the trans-Golgi network and in endocytic vesicles. Its cleavage substrates have arginine residues. It takes part in the stabilization of unstable cell surface MHC-I molecules in vesicular compartments derived from ER. This provides a second quality check-point for the loading of new peptides onto MHC molecules (Leonhardt *et al.*, 2010).

Cathepsins are cystein or aspartic-proteases located in endolysosomal compartments. Together with furin, cathepsins are involved through a vacuolar TAP-independent (TAP-ind) pathway in the generation of the epitope SIINFEKL from a chimeric fusion protein that links ovalbumin(OVA) and H-$2K^b$ that is internalized from the cell surface (Tiwari *et al.*, 2007).

### 2.1.2. ABC transporters.

The ATP-binding cassette (ABC) genes represent the largest family of transmembrane (TM) proteins. These proteins bind ATP to transport through extra- and intracellular membranes a wide diversity of compounds, such as metabolic products, lipids, drugs or peptides (Higgins, 2001; Childs and Ling, 1994).

The ATP-binding domain, also known as nucleotide-binding domain (NBD), contains two characteristic binding motifs (Walker A and B) separated by 90-120 aa. The functional protein contains two transmembrane domains (TMD) and two NBDs, commonly arranged as N-TMD-NBD-TMD-NBD-C. Some of the ABC genes encode half transporters, harboring only one TMD and one NBD. These gene products need to form homo- or heterodimers to conform a functional transporter. The TMD contains 6-11 α-helixes that provide specificity for the substrate. The NBDs confer the energy to the transport through membranes.

Phylogenetic analysis of the 49 human ABC genes places them into 7 subfamilies of proteins: ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG. ABC genes are expressed in all eukaryotic cells and are highly conserved between species. In the case of humans and mice, there is concordance between the two species, with some exceptions on the genes ABCB1 (duplication in mice, (Schinkel *et al.*, 1994; Schinkel *et al.*, 1997)); Abcg3 (present in mice but not in human, (Mickley *et al.*, 2001)); ABCC11 (loss in mice, (Tammur *et al.*, 2001); ABCA8 (duplication in mice (Annilo *et al.*, 2003)) and ABCA10 (loss in mice, (Annilo *et al.*, 2003)).

The ABCB family contains 11 genes, which are listed in **Table 2**.

| Gene name | Alias | Structure of the transporter | Transport function | References |
|---|---|---|---|---|
| **ABCB1** | PGY1, MDR | Full | Lipids, steroids and peptides (multidrug resistance) | (Gottesman *et al.*, 2002; Sharom, 2011) |
| **ABCB2** | TAP1 | Half (Heterodimer with TAP2) | Peptides | (Abele and Tampe, 1999) |
| **ABCB3** | TAP2 | Half (Heterodimer with TAP1) | Peptides | (Abele and Tampe, 1999) |
| **ABCB4** | PGY3 | Full | Phosphatidylcholine | (Ruetz and Gros, 1994; van Helvoort *et al.*, 1996) |
| **ABCB5** | | Full | Drugs | (Frank *et al.*, 2005) |
| **ABCB6** | MTABC3 | Half (Homodimer) | Fe/S cluster | (Mitsuhashi *et al.*, 2000) |
| **ABCB7** | | Half (Homodimer) | Fe/S cluster | (Csere *et al.*, 1998; Allikmets *et al.*, 1999) |
| **ABCB8** | MABC1 | Half (Homodimer) | Ions | (Ichikawa *et al.*, 2012) |
| **ABCB9** | TAPL | Half (Homodimer) | Peptides | (Wolters *et al.*, 2005; Demirel *et al.*, 2007) |
| **ABCB10** | MTABC2 | Half (Homodimer) | Unknown | (Liesa *et al.*, 2012) |
| **ABCB11** | BSEP, SPGP | Full | Bile salts | (Gerloff *et al.*, 1998) |

**Table 2. List of genes of the ABCB subfamily and their functions.**

Within this subfamily, only the heterodimer formed by TAP1 and TAP2 and TAPL are known to be involved in antigen presentation pathways (Abele and Tampe, 1999; Zhao *et al.*,

2006). Of note, although ABCB1 is one of the best studied genes in the subfamily, its function has always been related to multidrug resistance, especially in tumor cells (Shepherd *et al.*, 1993; Gottesman *et al.*, 2002; Sharom, 2011). However, there are reports characterizing its capability to transport peptides through the plasma membrane (Sharma *et al.*, 1992; Wang *et al.*, 2016).

#### 2.1.2.1. TAP.

TAP is a heterodimer formed by the gene products TAP1 and TAP2. The transporter is located in the ER membrane and it translocates peptides in a range of 8-16 aa from the cytosol to the ER lumen, whereas the most efficient transport is restricted to 8-12 aa peptides (Androlewicz and Cresswell, 1994; van Endert *et al.*, 1994).

TAP1 and TAP2 contain 10 and 9 transmembrane helixes, respectively. The last 6 helixes of each subunit form the core domain and are sufficient for peptide transport (Koch *et al.*, 2004). The remaining 4 and 3 TM N-terminal domains are needed for the recruitment of tapasin and the rest of the components of the PLC. The peptide-binding region is localized in the last cytosolic loop and a 15-aa region within the last TM region of TAP1 and TAP2 (Nijenhuis and Hammerling, 1996).The NBDs of both subunits are located in the cytosol, where they bind 2 ATP molecules by the Walker A and B loops from one subunit and the C loop from the other (**Figure 1**). The peptide binding step is ATP-independent (Androlewicz and Cresswell, 1994), whereas ATP hydrolysis is needed for the transport step (Neefjes *et al.*, 1993).

TAP affinity for the N-terminal aa of its substrates is different from the N-terminal affinity of MHC-I molecules, which suggests an important role of ER-resident aminopeptidases (van Endert *et al.*, 1995; Serwold *et al.*, 2002). The other selection criterion of TAP is the last C-terminal aa, which is preferably basic or hydrophobic (van Endert *et al.*, 1995).

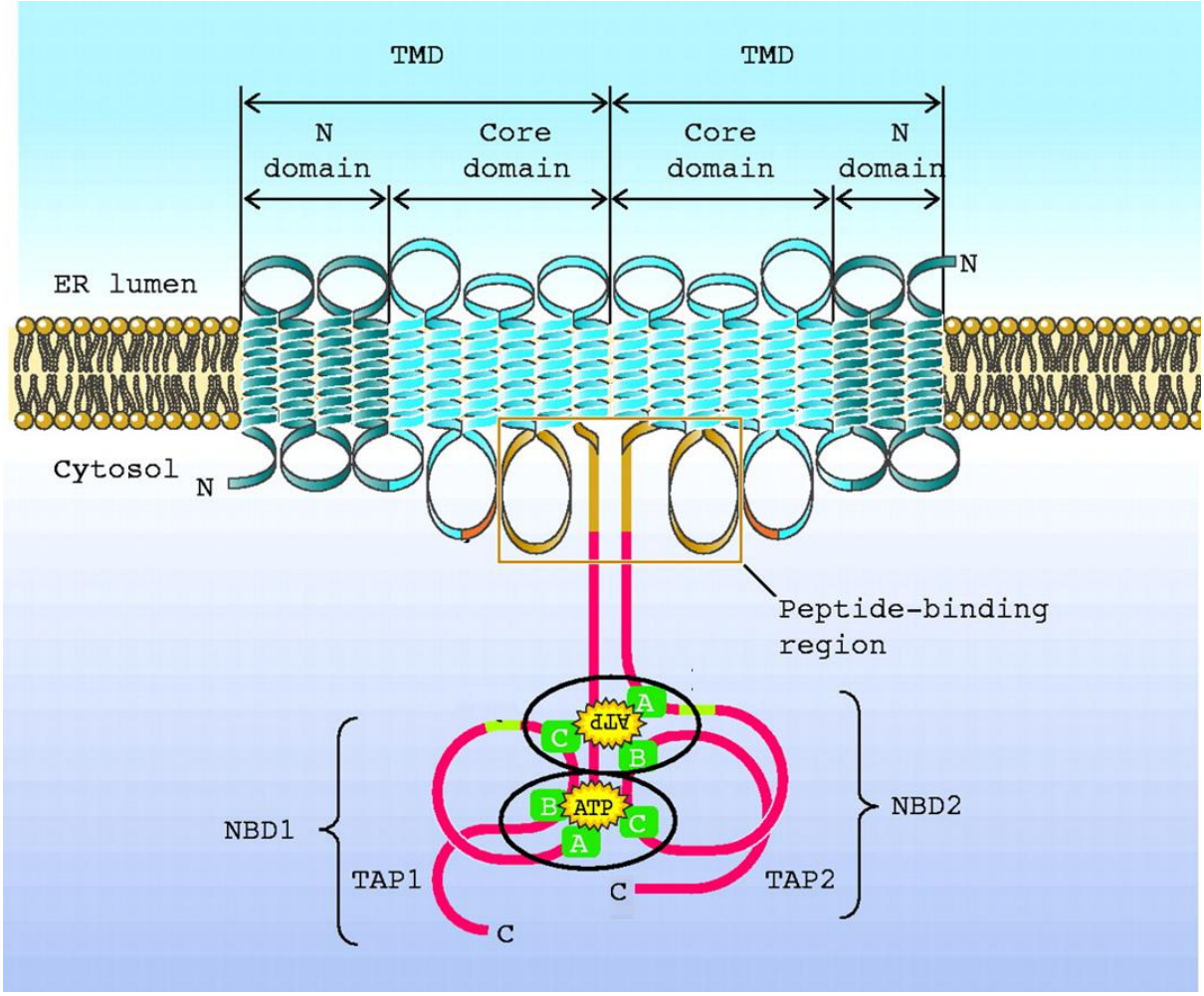



**Figure 1. Structure of TAP.**

The transporter TAP is formed by two subunits: TAP1 and TAP2. Each one of them contains 10 and 9 TM domains respectively, as well as a nucleotide-binding domain (NBD) in the cytosolic side of the ER membrane. The 6 C-terminal helixes of both subunits form the core domain, sufficient for peptide transport. The remaining N-terminal TM domains are necessary for the recruitment of PLC components. The NBDs are crucial for ATP binding, which will allow the transport of peptides through membranes (Abele and Tampé, 2004).

A correlation has been observed between the average length of the immunoproteasome products and TAP substrate selectivity, and also between TAP substrate sequences and MHC-I binding and T-lymphocyte recognition motifs (Uebel *et al.*, 1997). This establishes a scenario in which different proteins participate in the selection and edition of the MHC-I ligands (**Figure 2**), suggesting a coevolution of TAP with the other components of the classical antigen presentation route. This makes this transporter a key factor in the classical MHC-I antigen presentation route. However, TAP can potentially transport many diverse peptides (lineal, ramified or modified, up to 40 aa) to the ER lumen (Uebel *et al.*, 1995; Gromme and Neefjes, 2002). These peptides need to be edited in the ER for the

anchor to different MHC-I allotypes, opening a broad spectrum of possibilities for non-canonical antigen presentation routes.

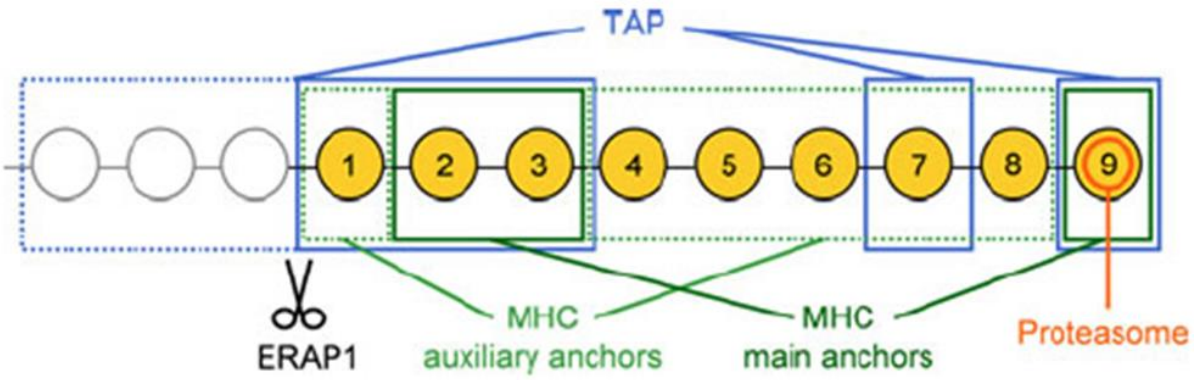



**Figure 2. Proteins involved in the selection and edition of canonical MHC-I ligands**.

In the canonical route of MHC-I antigen presentation, proteasomes generate the C-terminus of the peptides which are transported to the ER by TAP. ER-resident aminopeptidases like ERAP1 and ERAP2 trim the N-terminus of the peptides and make them suitable to anchor to MHC-I molecules (Mester et al., 2011).

In certain conditions, like in viral infections (Mayerhofer and Tampe, 2015), tumors (Leone *et al.*, 2013b) or TAP-deficient patients (Gadola *et al.*, 2000), TAP is absent ant the only antigen presentation available is TAP-independent antigen presentation. In these situations, the $CD8^{+}$T lymphocyte response may acquire a key role, since it is functional in the absence of TAP(Medina *et al.*, 2009).

#### 2.1.2.2. TAPL.

The TAP-like transporter (TAPL, ABCB9) is a homodimer that translocates peptides from the cytosol into the lumen of lysosomes. It presents certain homology with TAP1 and TAP2, with 39% of sequence identity with TAP1 and 41 % with TAP2, a similar identity to that between TAP1 and TAP2 (39 %) (Yamaguchi *et al.*, 1999). Its structure is similar to TAP's structure, with 10 α-helixes and one NBD. The 6 C-terminal helixes form the core of the transporter and show high identity with TAP1 and TAP2. The remaining 4 helixes show no identity to any protein in the databases. TAPL has a broad phylogenetic and tissue expression. It is speculated that TAPL gene is the ancestor of the ABCB family, since it is found in many taxa, even in invertebrates, and very conserved. Human and murine TAPL have a95 % identity (Zhang *et al.*, 2000), while TAP1 and TAP2 show 75 % conservation between rodents and humans(Kobayashi *et al.*, 2000).

Its tissue distribution is ubiquitous, although it has been found to be more abundant in testes, spinal cord and brain (Zhang *et al.*, 2000). Of note, its expression is upregulated in the differentiation of monocytes to dendritic cells, although a role in MHC-I antigen presentation has been discarded so far, since its expression does not restore MHC-I surface expression in TAP1- or TAP2-deficient cells (Demirel *et al.*, 2007). Although controversial in the beginning, TAPL subcellular localization is believed to be restricted to lysosomes, as established by immunofluorescence and subcellular fractionation (Zhang *et al.*, 2000; Demirel *et al.*, 2007; Demirel *et al.*, 2010).

In contrast to TAP, TAPL transports peptides with low affinity, making TAPL a very promiscuous transporter. Its substrates are in a range of 6-mer to 59-mer, with an optimum length of 23 aa. TAPL recognizes its peptides by the residues of both termini, preferring positively charged peptides with hydrophobic terminal residues, while negatively charged residues are disfavored (Wolters *et al.*, 2005).

Due to its ubiquous expression and high conservation in eukaryotes, TAPL has been proposed to be a housekeeping factor, preventing premature cell death by avoiding the accumulation of peptides in the cytosol (Bangert *et al.*, 2011). However, its overexpression in professional antigen presenting cells such as macrophages and dendritic cells suggests a role for TAPL in antigen presentation (Demirel *et al.*, 2007). It has been proposed to participate in autophagy, cross-presentation or negative selection of T-lymphocytes in the thymus (Bangert *et al.*, 2011), but it hasn't been observed any role in antigen presentation for TAPL so far.

### 2.1.3. Class I major histocompatibility complex molecules.

The function of MHC-I molecules is to bind peptides and present them on the cell surface to allow the screening of all the cells in the organism by $CD8^+$ T lymphocytes. The peptides displayed by MHC-I molecules on the cell surface are a representation of the inner status of a given cell. The recognition of viral or tumoral peptides by activated $CD8^+$ T lymphocytes leads to the elimination of that cell.

The structure of the MHC-I molecules consists of a heterodimer formed by a variant α chain and a non-variant β2-microglobulin (β2m). The α chain has three domains: α1 and α2, which form the groove that harbors the ligand, and α3, that interacts non-covalently with β2m. The α3 also anchors to the plasma membrane and interacts with the CD8 co-receptor of T lymphocytes, while the α1 and α2 together with the ligand are recognized by the TCR.

MHC-I molecules are polygenic (there are several MHC-I genes, and they are different in every individual) and highly polymorphic (there are multiple alleles of each MHC-I

gene within the population). These two features make it very difficult for a pathogen to escape the immune system. In humans, the genes encoding the MHC are located in the chromosome 6, and it contains more than 200 genes. These genes are called human leukocyte antigen (HLA). In mice, these genes are located in chromosome 17 and they are called histocompatibility 2 (H-2). There are 3 main MHC-I genes in both species: HLA-A, HLA-B and HLA-C in humans; and H-2K, H-2D and H-2L in mice. Each of these genes encode the α chain of the respective MHC-I protein. The β2m gene is located in human chromosome 15 and in murine chromosome 2. In different species, like mice, different strains have different haplotypes, which makes impossible for $CD8^+$ T lymphocytes from a given strain to recognize a different MHC-I/epitope complex from other strain of mice.

The binding of the ligand to the MHC-I groove is determined by much conserved positions in the MHC-I molecule. MHC-I molecules bind peptides of a small length-range, normally between 8 and 10 aa. However, it is possible for MHC-I molecules to bind larger peptides, protruding in the central region (Burrows *et al.*, 2007), in the N-terminus (Samino *et al.*, 2006) or in the C-terminus(Collins *et al.*, 1994). Each allotype has its own conserved residues, which provides each allotype with different peptide specificities (Falk *et al.*, 1991). The conserved motifs of each allotype are known for many human and mouse MHC-I molecules, which allows the prediction of epitopes of a given protein by different algorithms. The most popular tool for these predictions is SYFPEITHI (www.syphpeithi.de) (Rammensee *et al.*, 1999).

### 2.1.4. Cross-presentation.

Cross-presentation is defined as MHC-I presentation of antigen internalized by professional antigen presenting cells. In contrast, direct presentation deals with antigens synthesized within the cell. Cross-presentation plays a critical role in the $CD8^+$ T lymphocyte response to pathogens, in tolerance and in autoimmunity (Kurts *et al.*, 2010). Although most of the work concerning cross-presentation focuses on DCs, cells like macrophages, mast cells and epithelial cells can also cross-present antigens, being DCs the most efficient cross-presenting cells (Heath and Carbone, 2001). As reviewed (van Endert P., 2016), it is currently impossible to outline a well-defined pathway for cross-presentation, as most of the studies concerning MHC-I endocytic pathway have been done in non-professional antigen presenting cells (Donaldson and Williams, 2009). Cross-presentation is useful for $CD8^+$ T lymphocyte priming in three different contexts: (1) when pathogens do not infect directly DCs, the main APC that primes $CD8^+$ T lymphocytes, (2) when pathogens interfere with the endogenous MHC-I processing pathway and (3) when infected DCs die or lose function

rapidly after infection and only the uptake of their fragments by non-infected DCs can prime $CD8^+$ T lymphocytes.

It has been established that there are three potential pathways for cross-presentation (van Endert P., 2016).Antigens are internalized and can be translocated from endocytic vesicles to the cytosol. First, cytosolic peptides generated by proteasomes access the ER and bind nascent MHC-I molecules (**Figure 3; 1**). It has been shown that the ER-resident aminopeptidase ERAP1 is involved in cross-presentation of OVA/anti-OVA immunocomplexes while it is not required for soluble OVA cross-presentation (Firat *et al.*, 2007b). Second, cytosolic peptides can access endosomal compartments (**Figure 3; 2**) that receive ER components from ER-derived vesicles (Cebrian *et al.*, 2011). IRAP, resident in endocytic compartments, has been implicated in the cross-presentation of OVA-coated beads (Huppa *et al.*, 2010). The SNARE (Soluble N-ethylmaleimide-sensitive fusion protein-Attachment protein Receptor) protein Sec22b, located on the cytosolic side of the ER membrane, is crucial in these two pathways since it mediates fusion of ER-derived vesicles with phagosomes (Cebrian *et al.*, 2011). The last pathway does not require access of the internalized antigens to the cytosol and comprises the recycling of MHC-I molecules from the plasma membrane, involving lysosomal proteases (**Figure 3; 3**).

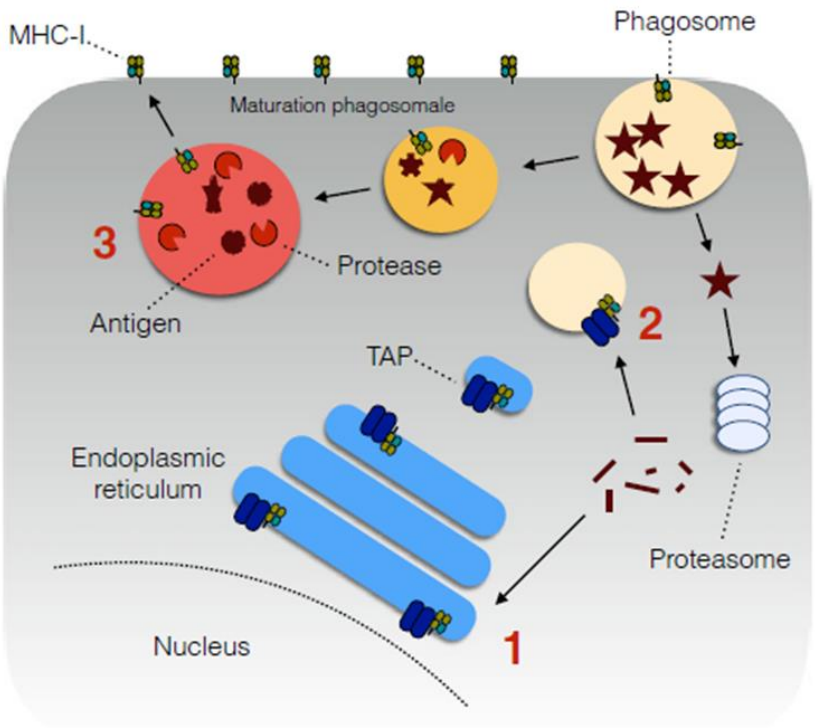



**Figure 3. Potential cross-presentation pathways.**

After internalization of the antigen and translocation to cytosol, preprocessed cytosolic peptides access either the ER (1) or specialized compartments resulting from the fusion of phagosomes and ER-derived vesicles (2). A third pathway involves recycling MHC-I molecules from the plasma membrane (3)(van Endert P., 2016).

### 2.1.5. Alternative direct antigen presentation routes.

Although TAP has a key role in MHC-I antigen presentation, it has been observed that some epitopes are presented on surface MHC-I molecules in the absence of TAP. These epitopes arise from many sources, such as viral proteins (Lee *et al.*, 1996; Khanna *et al.*, 1996; Sigal and Rock, 2000; Lautscham *et al.*, 2001), endogenous proteins (Gueguen *et al.*, 1994; Oliveira *et al.*, 2011) or minigene products (Anderson *et al.*, 1991; Raafat *et al.*, 2011). TAP1$^{-/-}$ mice show no defect in the expression of the TCR chains (Sandberg *et al.*, 1996), having the potential to recognize any epitope presented on MHC-I molecules. A broad and complete study of the contribution of TAP in the presentation of MHC-I-restricted epitopes encoded by a pathogen such as vaccinia virus has been done in our group, showing an unexpected high number of epitopes presented in VACV-infected dendritic cells lacking TAP: from 30 epitopes tested, 12 are presented in the absence of TAP (Lázaro S, PhD report).

The contribution of the proteasome, another key player in MHC-I antigen presentation, to the generation of TAP-independent epitopes has been observed in some individual epitopes, but most of them do not require this protease to be processed (reviewed in (Del Val *et al.*, 2013)). Both TAP-independent and proteasome-independent antigen presentation routes have been demonstrated as alternative pathways. It has been thought that both of them completely overlap, although that idea has been discarded with examples of TAP-independent epitopes that do require proteasome cleavage to be processed (Oliveira *et al.*, 2014; Merzougui *et al.*, 2011). A global study to determine how large this overlap is has not been done yet, and it is one of the goals of this work.

## 2.2. Activation and function of CD8$^+$ T lymphocytes.

Migratory dendritic cells (DC) are professional antigen-presenting cells (APC) that scan the organism seeking potential antigens. In the lymph nodes, these DC meet naïve CD8$^+$ T lymphocytes. If these naïve CD8$^+$ T lymphocytes recognize their specific antigen presented by the APC they get activated, a process known as priming. The recognized antigen can be originated from a self-protein expressed by the APC (direct presentation) or from an exogenous molecule endocytosed and processed by the APC (cross-presentation). If a CD8$^+$ T lymphocyte is activated by a cross-presented epitope, the priming is called cross-priming.

When they are activated, cytotoxic CD8$^+$ T lymphocytes (CTL) undergo a clonal expansion, generating an antigen-specific population that leaves the lymph node and migrates to the infection site attracted by chemokines produced in the infected tissue. When

the CTL recognize a target infected cell, they induce apoptosis on the latter by two cytotoxic mechanisms:(1) The secretion of granules harboring the protein perforin releases this protein, which integrates in the plasma membrane of the infected cell forming pores. Those granules also contain granzymes, a family of serine proteases that induce cell death of the infected cell by activating the caspase cascade. (2) The interaction of the Fas ligand (FasL) expressed on the $CD8^+$ T lymphocyte surface and its receptor Fas expressed on the infected cell surface. This interaction forms the death-inducing signaling complex (DISC) which triggers a signaling cascade in the infected cell that ultimately activates caspase 8. These proteases provoke the death of the infected cell.

When the population of infected cells is cleared, the number of $CD8^+$ T lymphocytes is substantially reduced. Some of the CTL that got primed in the lymph nodes do not differentiate to effector CTL, but they acquire a memory $CD8^+$ T lymphocyte phenotype, which allows a more rapid and specific response against a potential subsequent infection by the same pathogen.

## 2.3. Vaccinia virus.

Vaccinia virus (VACV) is a large, enveloped double-stranded DNA virus that contains more than 200,000 base pairs that encode more than 200 proteins. It belongs to the Orthopoxvirus family, which includes a large number of viruses that infect different species like rabbits, camels or monkeys. Variola virus, the virus that causes smallpox in humans, also belongs to this family. It was eradicated during the 1970 decade by using VACV infections as a vaccine, which granted it its name. The high efficiency of VACV as a vaccine against the variola virus suggests a strong identity of antigenic peptides between these two viruses. The origin of VACV is still controversial, but it is thought to derive from the cowpox or the horsepox viruses (Baxby, 1977; Turner, 1982; Tulman *et al.*, 2006). Its long history in vaccination has made VACV the model poxvirus in the laboratories. As a consequence, many strains of VACV arose.

### 2.3.1. Virion structure.

The VACV mature virion (MV) has a barrel shaped structure with a nucleoprotein core flanked by protein structures called lateral bodies and an outer single bilayer lipoprotein envelope (Hollinshead *et al.*, 1999) (**Figure 4**). Purified MVs contain at least 80 proteins (Chung *et al.*, 2006). MV envelope seems to derive from the ER membrane modified with viral proteins (Moss, 2015). During VACV morphogenesis, the MV is wrapped by two more membranes coming either from the trans-Golgi or the endosomal vesicles (Hiller and Weber,

1985; Tooze *et al.*, 1993). This is the extracellular virion (EV), which is released from the cell by exocytosis, unlike the MV which is released by cell lysis. Both virions have infective capacity.

### 2.3.2. Life cycle

The first step to infect a cell is the entry of the virus. This step itself shows much variation among virus strains and cell types, observing from endocytosis of the viral particles to membrane fusion (Bengali *et al.*, 2011; Bengali *et al.*, 2012). The strain western reserve (WR) uses a low-pH endocytic pathway to enter the target cell (Bengali *et al.*, 2009). When internalized, the nucleoprotein core is released to the cytoplasm, where the viral DNA is accessible for transcription of viral early genes. It is in the cytoplasm where viral factories are formed. These structures are cytoplasmic domains transiently surrounded by ER-derived cisternae (Tolonen *et al.*, 2001) where viral DNA is concentrated. One characteristic and distinguishable structure of the viral factories is the crescent. The crescents are lamellar bodies derived from ER-cisternae(Husain *et al.*, 2006; Maruri-Avidal *et al.*, 2011) with a scaffold of trimers of the viral protein D13 (Szajner *et al.*, 2005). After crescents are formed, viral DNA replication and virion assembly take place in these factories(Schramm and Locker, 2005; de Castro *et al.*, 2013).The crescent remains with the immature virion, D13 scaffold is degraded and core condensation progresses(Liu *et al.*, 2014). The MV can be then wrapped by a double membrane coming from the trans-Golgi or the endosomal compartments to form the wrapped virion (WV) with three lipid bilayers. When leaving the cell by exocytosis, the WV loses one of the three bilayers and forms the EV, with two lipid bilayers (**Figure 4**).

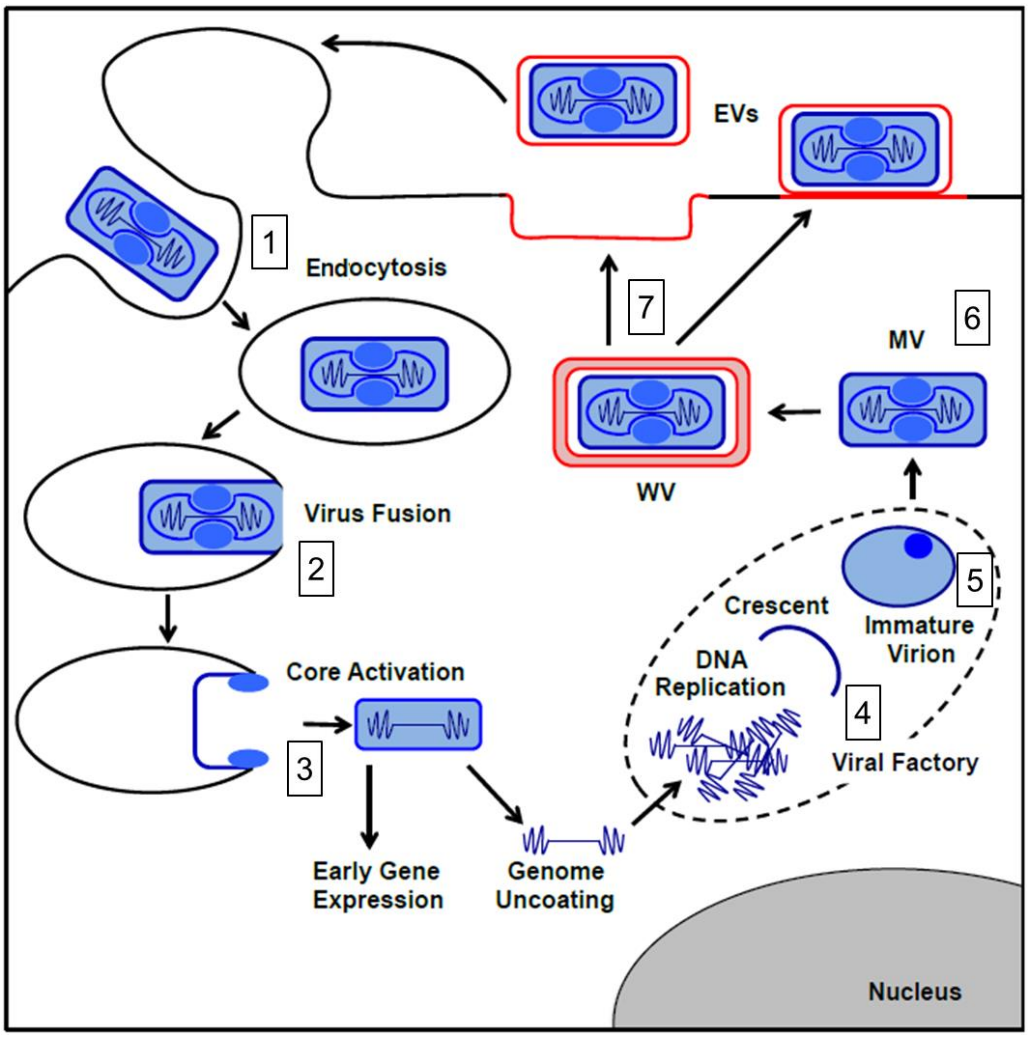



**Figure 4. VACV life cycle.**

The VACV virion enters the cell either by membrane fusion (for example, the strain Copenhagen) or by endocytosis (strain WR) (1). Then, the nucleoprotein core is released to the cytoplasm by the fusion of the virion membrane with the endocytic vesicle membrane (2). The transcription of early-expression genes takes place in the cytosol before the formation of viral factories (3). In the viral factories, DNA replication, the transcription of post-replicative-expression genes, the formation of the crescent (4) and the immature virion formation take place (5). D13 is degraded, core condensation progresses and the mature virion (MV) is released out of the viral factories (6). The MV is wrapped by double cellular membranes to form the wrapped virion (WV), which is transported to the plasma membrane to leave the cell by exocytosis, forming the extracellular virion (EV) (7). Adapted (Bidgood and Mercer, 2015).

### 2.3.3. Gene expression kinetics.

Unlike most viruses, poxviruses replicate and transcribe their DNA in the cytosol rather than in the nucleus of the host cell. Their different open reading frames (ORFs) are closely spaced and temporally regulated. Early-expressed genes do not require DNA replication to be expressed, while intermediate- and late-expressed genes have a post-

replicative (PR) expression. There is a cascade mechanism of regulation by which early genes are transcribed by late transcription factors, intermediate genes are transcribed by early transcription factors and late genes are transcribed by intermediate transcription factors (Baldick, Jr. and Moss, 1993; Davison and Moss, 1989; Keck *et al.*, 1990) (**Figure 5**). Within the whole genome of VACV, 118 early genes, 53 intermediate genes and 33 late genes have been found (Yang *et al.*, 2011). When the nucleoprotein core reaches the cytoplasm, early mRNAs contained in the virion are released into the cytoplasm, where they are translated. Early proteins can be divided according to their function into enzymatic proteins required for DNA replication (such as the DNA polymerase), transcription factors required for the transcription of intermediate genes and structural proteins, required for the morphogenesis of new virions (**Figure 5**). When the DNA is replicated, PR expression genes (intermediate and late) can be transcribed and translated. Treatments that impair this replication step inhibit the expression of both intermediate and late genes, like the treatment with the DNA replication inhibitor cytosine arabinoside (AraC) (Yang *et al.*, 2011) or with proteasome inhibitors (Satheshkumar *et al.*, 2009). The three categories of genes encode structural gene products necessary for the assembly of the immature virion (IV) (**Figure 5**).

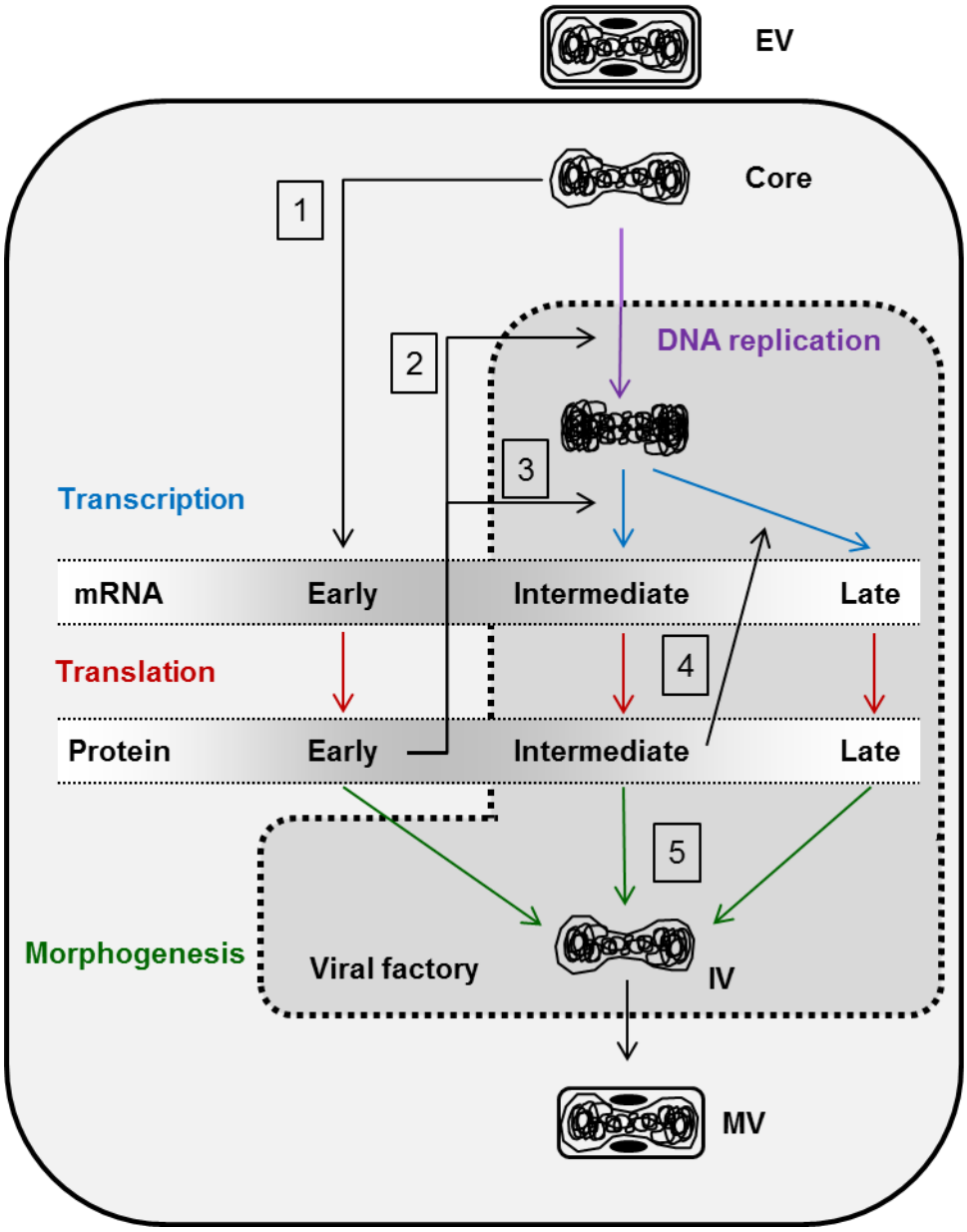



**Figure 5. Gene expression kinetics of VACV.**

After entry of the extracellular virion (EV) or mature virion (MV), the nucleoprotein core releases early mRNAs into the cytoplasm (1). Early mRNAs are translated, which allows the DNA replication in the viral factories by early gene products like DNA polymerase (2). Early transcription factors bind to the replicated DNA to transcribe intermediate genes (3). Likewise, intermediate transcription factors trigger the transcription of late genes (4). The structural proteins coming from early, intermediate and late genes together with replicated DNA form new immature virions (IV) (5) that will leave the viral factory as mature virions (MV). Based on (Moss, 2001).

### 2.3.4. Immune response against VACV.

Although there are some studies in non-human primates regarding the immune response against VACV (Earl *et al.*, 2004; Edghill-Smith *et al.*, 2005), most of the work on

this issue has been done in mice. However, it must be considered that this species is not the natural host of the virus and that there are some differences in the infection between mice and humans, like the tropism of the virus or the affinity of the immunomodulatory proteins encoded by the virus. One example is the viral protein B8R, a soluble IFNγ receptor that binds human IFNγ but not murine IFNγ (Mossman *et al.*, 1995; Alcami and Smith, 1995). This agrees with the observation that a rVACV lacking B8R shows similar virulence compared to the WT virus in infected mice (Verardi *et al.*, 2001).

The innate cellular response against VACV comprises macrophages, neutrophils and natural killer (NK) cells. Macrophages have been found infiltrated in the site of infection in early days post-infection (d.p.i.) in mice (Reading and Smith, 2003; Jacobs *et al.*, 2006). Although infection by VACV of macrophages isolated from mice has been observed to be abortive (Broder *et al.*, 1994), it has been found that human monocyte-derived macrophages are permissive to VACV infection, and it is suggested that they participate in virus dissemination during the infection (Byrd *et al.*, 2014). Whether there is a role for macrophages in the response against VACV and the nature of this response remains unknown. Neutrophils participate in the primary response against the virus by phagocytizing extracellular virions and degrading them (West *et al.*, 1987; Jones, 1982).Since VACV can spread in a cell-to-cell way (Moss, 2006), the response against extracellular virions is not sufficient to fight a VACV infection. NK cells can develop a cytotoxic response against infected cells. These cells rapidly migrate to the site of VACV infection (Natuk and Welsh, 1987). Murine and human NK cells show the capability to recognize and kill VACV-infected cells (Brutkiewicz *et al.*, 1992), as well as in the human system (Chisholm and Reyburn, 2006). Yet, the exact role of NK cells in primary VACV infections remains unclear.

The humoral response against VACV is an important component of the fight against the virus, as VACV-infected mice lacking B lymphocytes show viral titers increased up to 100-fold when compared to WT infected mice(Xu *et al.*, 2004). However, it has been observed that the antibody response against VACV prevents the dissemination of the virus, but it does not protect from mortality (Mota *et al.*, 2011).

$CD4^+$ and $CD8^+$ T lymphocytes take part on the cellular adaptive response against VACV. There is no evidence that $CD4^+$ T lymphocytes exhibit direct antiviral response, although mice lacking $CD4^+$ T lymphocytes show increased viral titters (Xu *et al.*, 2004). $CD4^+$ T lymphocytes are an important source of IFNγ, a key cytokine for the activation of $CD8^+$ T lymphocytes. There is also a correlation between the accumulation of $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes in the site in infection (Jacobs *et al.*, 2006). $CD4^+$ T lymphocytes also provide help for antibody response against VACV (Sette *et al.*, 2008). $CD8^+$

T lymphocyte function appears to be vital for the protective response against VACV (Wherry *et al.*, 2001; Amanna *et al.*, 2006). It has been observed that direct antigen presentation is sufficient for the activation of VACV-specific $CD8^+$ T lymphocytes (Xu *et al.*, 2010), although for the non-replicative VACV strain MVA, cross-presentation appears to be crucial for this activation (Gasteiger *et al.*, 2007). There is a large expansion of VACV-specific $CD8^+$ T lymphocytes following infection (Harrington *et al.*, 2002). When $CD8^+$ T lymphocytes are depleted *in vivo* with antibody, $CD8^+$-depleted VACV-infected mice show higher viral titers (from 2-fold to 10-fold) than control mice. However $CD8^+$-depleted VACV-infected mice show higher weight loss than control mice, but all of them recover (Xu *et al.*, 2004). This shows the existence of non-$CD8^+$-mediated mechanisms in the protection against this virus. A similar result is obtained when mice lacking humoral response are infected, but when mice are deprived of both humoral and $CD8^+$ T lymphocyte action, they never recover from VACV-induced lesions (Xu *et al.*, 2004). Therefore, there is a collaborative action of different immune mechanisms to control a VACV infection.

### 2.3.5. Antigen presentation of VACV epitopes to $CD8^+$ lymphocytes.

Within 24 hours after an i.p. infection, VACV is spread throughout the organism (Lin *et al.*, 2013). When the virus reaches its target cells, these are infected and they start to process antigens to present epitopes recognized by $CD8^+$ T lymphocytes on their surface. It has been demonstrated that for 6 out of 8 epitopes studied, their source is the new synthesis of proteins (presumably DRiPs), instead of post-translational proteins, as the time in that the epitope is detected matches the time of detection of the corresponding protein(Croft *et al.*, 2013). The discovery of a VACV promoter that drives very early gene expression has led to the division of the early gene expression into E1.1 (very early) and E1.2 (early) (Wennier *et al.*, 2013). This subclassification is supported by proteomics analyses that show that there are indeed two clusters of VACV genes, whose expression is resistant to the DNA replication inhibitor AraC (Yang *et al.*, 2010; Croft *et al.*, 2015). These analyses have also observed that there is a good correlation between gene transcription and protein detection and between gene expression and protein function, as early-expressed proteins are involved in DNA replication and host interaction, followed by genes involved in transcription and virion components, which are expressed at later times.

Many techniques and approaches have been used to determine the VACV antigens recognized by $CD8^+$ T lymphocytes, like predictive algorithms, chromatography and mass spectrometry, peptide libraries, etc. It has been proposed that mRNA levels can predict immunoprevalence of their corresponding proteins for $CD8^+$ T lymphocyte response (Sette *et al.*, 2009). However, there are many factor that determine the selection of antigens, such as

cellular processing, MHC-I binding, TCR recognition or immunomodulation (Sette *et al.*, 2009), and their prediction cannot be that simplistic (Croft *et al.*, 2013). Until now, more than 200 antigenic determinants for $CD8^+$ T lymphocytes have been described in 7 different human allotypes (Oseroff *et al.*, 2005; Pasquetto *et al.*, 2005; Terajima *et al.*, 2006) and 5 murine allotypes together. In the BALB/c strain, 54 determinants have been described presented by the MHC-I molecules H-$2K^d$, H-$2L^d$ and H-$2D^d$ (Tscharke *et al.*, 2006; Oseroff *et al.*, 2008). In the C57BL/6 strain, 49 antigenic determinants have been found, presented by H-$2K^b$ and H-$2D^b$ molecules (Tscharke *et al.*, 2005; Moutaftsi *et al.*, 2006).

There is an immunodominance hierarchy determined by how abundant the $CD8^+$ T lymphocyte response is against every single epitope (Moutaftsi *et al.*, 2006). In this hierarchy in C57BL/6 mice, the epitope B8R accounts for about 25 % of the global response against VACV (Moutaftsi *et al.*, 2006). This immunodominance can be altered depending on the route of administration of the virus, being the difference between B8R and the rest of the VACV epitopes more acute for systemic routes (i.v. and i.p.) (Lin *et al.*, 2013). However, this hierarchy does not correlate with the protection hierarchy, obtained when C57BL/6 mice are vaccinated with a VACV epitope followed a VACV challenge, showing that immunodominance does not predict protective capacity of VACV epitopes (Moutaftsi *et al.*, 2009). The percentage of $CD8^+$ T lymphocytes that recognize VACV epitopes in a VACV infection has been estimated to be 60 % of the total $CD8^+$ lymphocyte population (Yuen *et al.*, 2010).

If mice are primed with a single VACV epitope, the $CD8^+$ T lympchocyte response to it after a following VACV challenge is greatly enhanced. However, this enhanced response is only detected several weeks after the challenge (Russell *et al.*, 2017). The priming with single epitopes does not alter the response to the rest of the viral epitopes, maintaining the immunodominance hierarchy (Russell *et al.*, 2017).

Of note, previous results in our lab show that, surprisingly, most of the VACV epitopes studied (15 out of 24), follow alternative antigen processing pathways, either TAP-independent (12) or proteasome inhibitor-resistant (3). Also, most of the TAP-independent VACV epitopes characterized need proteasome cleavage, in contrast with the previously described in the bibliography (reviewed in (Del Val *et al.*, 2013)). Altogether, this results show the high prevalence of antigen presentation pathways and therefore, the need of studying these alternative antigen presentation pathways in depth.

## 3. OBJECTIVES.

With the aim of studying TAP-independent (TAP-ind) viral antigen processing routes for the presentation by MHC-I molecules, we analyzed the response of $CD8^+$ T lymphocytes from C57BL/6 mice against VACV-infected antigen presenting cells. We formulated the following objectives:

1. To study the involvement of Sec22b in TAP-independent antigen cross-presentation routes.
2. To seek alternative transporters for TAP-independent VACV epitopes.
3. To quantify the participation of proteasomes to the generation of VACV TAP-independent epitopes.
4. To analyze the role of non-proteasomal proteases in the generation of VACV epitopes.
5. To study the physiological relevance of alternative antigen presentation pathways by analyzing the protective capacity of TAP-independent epitopes against a VACV infection *in vivo*.
6. To find common and distinctive characteristics of TAP-independent VACV epitopes and/or potential precursors.

# 4. MATERIALS AND METHODS.

## 4.1. Reagents

### 4.1.1. Culture media.

The culture media used in this work are the following:

-DMEM: Dulbecco-modified Eagle medium supplemented with ultraglutamine (Lonza) and 10 % fetal bovine serum (FBS) (Sigma).

-RPMI: Roswell Park Memorial Institute 1640 medium supplemented with ultraglutamine (Lonza), $5 \cdot 10^{-5}$ M β-mercaptoethanol (Merck) and 10 % FBS.

-α-MEM: Minimum essential medium α supplemented with ultraglutamine (Gibco), $5 \cdot 10^{-5}$ M β-mercaptoethanol, 100 U/mL penicillin (Sigma), 100 U/mL streptomycin (Sigma), 10 µg/mL kanamycin (Sigma), 2.5 µg/mL amphotericin (Sigma) and 3 % FBS.

-IMDM: Iscove's Modified Dulbecco's Medium (Sigma) supplemented with 2 mM L-alanyl-L-glutamine (GlutaMAX$^{TM}$, Gibco), $5 \cdot 10^{-5}$ M β-mercaptoethanol, 10 % FBS, 100 U/mL penicillin (Sigma) and 100 U/mL streptomycin (Sigma) was used to culture BMDCs in the lentiviral transduction protocol.

-Opti-MEM (Gibco) was used for transfection of HEK 293T cells.

-Trypsin-EDTA: Trypsin (Sigma) diluted in EDTA 0.5 M pH 8 (Merck) was used to subculture adherent cells.

-PBS: Phosphate-Buffered Saline: 137 mM NaCl, 1.5 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$ and 2.7 mM KCl.

### 4.1.2. Chemical products.

Inorganic salts, acids, bases and inorganic compounds such as methanol or ethanol were mostly acquired from Merck. Sucrose and bovine serum albumin (BSA) were also supplied by Merck.

Paraformaldehyde (PFA) was supplied by Electron Microscopy Sciences.

Sigma provided dimethyl sulfoxide,β-mercaptoethanol, crystal violet, polybrene and brefeldin A (BFA).

Lipofectamine and PLUS reagent for cell transfection were supplied by Life Technologies.

The soluble protein OVA was acquired from Worthington Biochemical Corporation.

The protease inhibitors used are summarized in **Table 6**. Lactacystin was supplied by E. J. Corey (Harvard University, Cambridge, MA, U.S.A.), leucinthiol, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) and decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-cmk) were supplied by Bachem. Captopril was acquired from Sigma.

Recombinant human interleukin 2 (IL-2) was a kind gift from the NCI Preclinical Repository from the National Institutes of Health from the U.S.A.

The LIVE/DEAD Fixable Violet Dead Cell Stain Kit used to stain dead cells was supplied by ThermoFisher Scientific.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) used to differentiate bone marrow precursors into dendritic cells in culture was a supernatant generated in the lab by culturing the J558 cell line.

### 4.1.3. Antibodies.

The antibodies used in flow cytometry for the intracellular cytokine staining anti-mouse CD8β.2 (53-5.8 clone) conjugated with fluorescein isothiocyanate and anti-mouse interferon-γ (XMG1.2 clone) conjugated with phycoerythrin, diluted respectively 1/300 and 1/200 were supplied by Biolegend. The antibody anti-mouse H-2K$^{b}$ conjugated with phycoerythrin was supplied by BD Biosciences and was diluted 1/300. The antibody anti-CD11c conjugated with phycoerythrin was supplied by eBiosciences and the antibody anti-GR1 conjugated with allophycocyanin was supplied by Biolegend and both were diluted 1/300.

The primary antibodies used for the immunoblotting assay were the following: rabbit polyclonal anti-TAPL (D01P, Abnova) diluted 1/100, rabbit polyclonal anti-Tubulin (Sigma) diluted 1/500, rabbit polyclonal anti-Sec22b(Synaptic Systems) diluted 1/200, rabbit anti-ERp57 (#2890 Ted H. Hansen (Harris et al., 2001)) diluted 1/200, rabbit anti-Tapasin (#2668 Ted H. Hansen (Harris et al., 2001)) diluted 1/300 and mouse monoclonal anti-Actin (AC-40, Sigma) diluted 1/500. The secondary antibody used was goat anti-rabbit HRP (SouthernBiotech) diluted 1/5000.

CD8$^{+}$ T lymphocytes were purified by negative selection using the mouse CD8$^{+}$ T cell isolation Kit (MACS, Miltenyi Biotech).

### 4.1.4. Peptides.

The peptides were synthetized in an Applied Biosystems peptide synthesizer (model 433A) and purified. The identity was confirmed by mass spectrometry in a Brücker Dalthonics MALDI-TOF equipment (model REFLEX) by Dr. D. López (Proteomics Unit, Centro Nacional de Microbiología, Instituto de Salud Carlos III,Madrid). Homogeneity of all peptides was checked by reversed-phase high resolution liquid chromatography in a BIOCAD equipment (Applera).

The synthetic peptides of VACV harbor the same aminoacidic sequences of the viral epitopes described (Moutaftsi *et al.*, 2006). They were named after the coding gene. When more than one epitope in one protein is studied, the starting position of the epitope in the protein is indicated after the gene name. The one-letter code is used to name aa. The characteristics of the epitopes and the proteins they derive from are summarized later in **Table *9***.

The peptide SIINFEKL is harbored by the protein Ovalbumin (OVA) in the positions 257-264.

### 4.1.5. Cell lines and primary cells.

-CV1 cells: Adherent fibroblasts from African green monkey kidney (ATCC: CCL-70). The culture medium used for these cells was DMEM.

-HEK 293T: *Homo sapiens* embryonic kidney 293T cells containing the SV40 large T-antigen (ATCC: CRL-3216). They are adherent cells and were cultured in DMEM.

-RMA: A subline of the Rauscher virus-induced T cell lymphoma RBL-5 ($H\text{-}2^b$) (Ljunggren and Karre, 1985). They grow in suspension and were cultured in DMEM.

-RMA-S: A TAP2-deficient variant of the RMA cell line (Ljunggren *et al.*, 1991; Zhou *et al.*, 1993). They are adherent and were cultured in DMEM.

-B3Z: A T-cell hybridoma engineered to secrete β-galactosidase when its T-cell receptor engages an $OVA_{257\text{-}264}/K^b$ complex (Karttunen *et al.*, 1992). They are non-adherent cells and were cultured in RPMI.

-J558: A murine B lymphocyte myeloma (ATCC: TIB-6). They grow in suspension and were cultured in RPMI. They were used to obtain in their supernatant the GM-CSF that differentiates the bone marrow precursors into dendritic cells.

-Bone marrow-derived dendritic cells (BMDC): Bone marrows were obtained from femurs of healthy C57BL/6 mice. Bone marrow precursors were cultured in RPMI supplemented with 10 % FBS, antibiotics and 10 % GM-CSF-enriched medium from J558 cells at a concentration of $1 \cdot 10^6$ cells/mL. The same volume of the same composition was added again 3 and 6 days after the culture setting. Only the non-adherent cells were used for the experiments. For the vaccination assays, BMDC were matured incubating for 1 h in medium with 0.5 µg/mL of lipopolysaccharide (LPS). The $CD11^+/GR1^-$ phenotype of the BMDC was checked by flow cytometry at day 7.

-Monospecific polyclonal $CD8^+$cytotoxic T lymphocytes (CTL): Their obtention and establishment is described in the $CD8^+$ T lymphocyte section 4.4. They were cultured in α-MEM supplemented with 3 % FBS.

All cells were cultured at 37 ºC in a 98% humidity atmosphere. Cells cultured with RPMI medium were cultured in a 5 % $CO_2$ atmosphere. The rest of the cells were cultured in a 7 % $CO_2$ atmosphere.

#### 4.1.6. Viruses.

The strain used in most of the infections was Western Reserve (WR). We used the recombinant VACV expressing full-length OVA (rVACV-OVA), derived from the strain WR for infection of BMDC in lactacystin assays to use the presentation of the epitope SIINFEKL as a control, since it has been reported that the presentation of $K^b$/SIINFEKL complexes in cells infected with recombinant VACV expressing full-length cytosolic OVA shows high sensitivity to this proteasome inhibitor (Antón *et al.*, 1998). When the expression of the green fluorescent protein (GFP) was needed, we used the GFP-expressing recombinant vaccinia virus recombinant VACV expressing GFP (rVV-GFP).

## 4.2. Mice.

The following strains of mice were used in this work:

-C57BL/6JRccHsd (C57BL/6 from now on) used as a WT control (H-$2^b$ haplotype).

-BALB/c (H-$2^d$ haplotype).

-TAP1$^{-/-}$ mice (B6.129S2-Tap1tm1Arp/J; #002944, Jackson) (H-$2^b$ haplotype). In these mice, 7 Kb from the TAP1 gene have been replaced by a neomycin resistance gene, encompassing about 80 % of the TAP1 protein-encoding region(van Kaer *et al.*, 1992).Lack of expression of TAP1 leads to lack of assembly and degradation of TAP2(Furukawa *et al.*, 1999).

Mice were immunized and infected intraperitoneally. For immunizations, BMDC were matured with LPS and incubated with $10^{-6}$ M synthetic peptide, and $1 \cdot 10^6$ of BMDC per peptide were injected, never exceeding $6 \cdot 10^6$BMDC per mouse. For infections with VACV, $5 \cdot 10^6$plaque-forming units (PFU) of virus were administered. Mice were sacrificed in a $CO_2$ atmosphere.

All mice were kept under pathogen-free conditions and studies were approved by the CSIC Animal Care Committee, with reference PROEX 302/14. All experiments were performed in accordance with relevant guidelines and regulations.

## 4.3. Viral titration and viral infections.

### 4.3.1. Production, purification and titration of virus.

Vaccinia viruses were grown in CV1 cells and purified from cell homogenates by ultracentrifugation in a sucrose cushion (Johnstone *et al.*, 2004). Viral titration was assayed by serial dilutions, inoculation of CV1 cells and crystal violet staining 24 hours post-infection. The viral titer is expressed as plaque-forming units per milliliter (PFU/mL).

### 4.3.2. Viral titration of organs.

Spleens were harvested in 1 ml of PBS from mice three days after viral infection and kept frozen for no more than one week at -80ºC. They were thawed and homogenized by centrifugation with ceramic beads in a MagNA Lyser instrument (Roche) in a cold room at 4 ºC. Ovaries were extracted and each frozen in 1 mL of PBS at -80 ºC. Homogenization was carried out by mechanic methods and 3 series of freeze-thaw cycles. The resulting lysates were tittered following the regular protocol starting at the minimum dilution 1/10.

### 4.3.3. Viral infections.

BMDC were infected with VACV according to previously described protocols (Eisenlohr *et al.*, 1992) with some modifications. Viral adsorption was performed at 4 ºC in PBS with 0.1 % BSA at a cell concentration of $1 \cdot 10^7$ cells/mL. The multiplicity of infection (m.o.i.) used in the experiments was in a range of 3-10, generally 5. After washing the cells, they were resuspended in culture medium at a concentration of $1 \cdot 10^6$ cells/mL and incubated at 37 ºC from 6 to 16 h in order to proceed with the infection.

In the case of inhibitor treatment, the infection never exceeded 6 h and the chemicals were added during the adsorption and added again at the desired concentration during the infection.

## 4.4. Effector cells.

Different sources of effector cells were used:

-Purified $CD8^+$ T lymphocytes: To study global viral antigen presentation using VACV-infected BMDC, we purified $CD8^+$ T lymphocytes from spleens of infected C57BL/6 mice. The purification is achieved by negative selection of $CD8^+$ T lymphocytes using a cocktail of biotin-conjugated antibodies against CD4, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC-class II, Ter-119 and TCRγ/δ. The isolation of T cells is achieved by depletion of magnetically labeled cells. After the incubation with the cocktail, splenocytes were incubated with anti-biotin antibody-conjugated magnetic beads. Non-bound $CD8^+$ T lymphocytes were collected by elution through a magnetized column. The process achieves a purification of at least 95 %.

-Peritoneal exudate cells (PEC): To study the recognition of individual peptides after intraperitoneal vaccination with BMDC loaded with peptides followed by VACV challenge, PEC containing $CD8^+$ T lymphocytes were used. One million PEC were incubated in the presence of BFA with a saturating concentration of each synthetic peptide for 6 h to stimulate their $CD8^+$ T lymphocytes.

-Monospecific Cytotoxic T Lymphocytes (CTL): To study the processing and presentation of individual epitopes, we generated polyclonal monospecific CTL lines from spleens from infected C57BL/6 mice as described before (López and Del Val, 1997). Briefly, mice were infected twice 21 days apart with $5 \cdot 10^6$PFU of VACV (WR or rVACV-OVA). Twenty-one days after the boost, spleens were harvested and splenocytes were cultured in multiwell plates in the presence of the synthetic peptide of interest in an optimized concentration, different for each peptide (from $10^{-8}$ to $10^{-10}$ M). Five days later, 25 U/mL IL-2 was added. The expansion and maintenance of the line was achieved by weekly stimulation with splenocytes loaded with the peptide of interest at the same concentration and with IL-2.

-B3Z: To study the cross-presentation of OVA, we used the B3Z T-cell hybridoma that specifically and exclusively recognized the $OVA_{257-264}/K^b$ complex.

## 4.5. Intracellular cytokine staining assay.

The intracellular cytokine staining assays (ICS) measure the activation of T lymphocytes after recognition of antigen presented by an infected cell or antigen presenting cell. They were performed according to previously described methods (Chen *et al.*, 2000; Johnstone *et al.*, 2004). In particular, PEC were stimulated with synthetic peptides in a saturating concentration ($10^{-6}$ M) for 6 h and BFA was added for the last 5 h. Purified $CD8^+$ T

lymphocytes were stimulated for 6 h in the presence of BFA since the beginning of the stimulation or 2 h after incubation with infected BMDC at serial Effector:Target (E:T) ratios in a range of 1:3 to 1:30. CTL were stimulated in the presence of BFA for 4 h with infected BMDC at an E:T ratio of 1:5 or with serial concentrations of synthetic peptides ($10^{-11}$ to $10^{-8}$ M) to test their sensitivity. After stimulation, $CD8^+$ T lymphocytes were fixed, permeabilized and stained with anti-CD8-FITC and anti-IFNγ-PE antibodies. As an alternative for the fixating and permeabilizing reagents of the IntraStain kit for cytometry (Dako), we used a fixating solution containing PBS with 4 % PFA and a permeabilizing solution containing PBS with 4 % PFA, 0.1 % saponin and 1 % BSA. Cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences) equipment to detect the stained cells and the percentage of $IFN\gamma^+$ cells within the $CD8^+$ population was calculated using the FlowJo V10 software.

## 4.6. Cross-presentation assay.

BMDC were cultured in flat-bottomed 96-well plates at a concentration of $1{\cdot}10^5$ cells/200 µl. Three µm latex beads (17134, Polysciences) were coated with OVA and BSA at different proportions by incubating the beads with the proteins rotating at 4 ºC o.n. The following day, after washing, 200 beads/cell were added to the BMDC presenting cells and after a 5 h incubation at 37 ºC, cells were washed and fixed with 0.008 % glutaraldehyde in PBS by incubating at 4º C for 3 min. To stop the fixation, 0.4 M glycine was added. After several washes, $1{\cdot}10^5$OVA-specific B3Z T cells per well were added and recognition of the antigen was allowed by incubating at 37 ºC. After 16h, T cell activation was measured detecting β-galactosidase activity by optical density at 590nm (Victor Wallac, Perkin Elmer) using chlorophenol red-β-D-galactopyranoside (CPRG) in LacZ buffer (9 mM $MgCl_2$, 0.125 % NP40 in PBS) as substrate for the reaction.

## 4.7. Generation of lentiviral particles and lentiviral transduction.

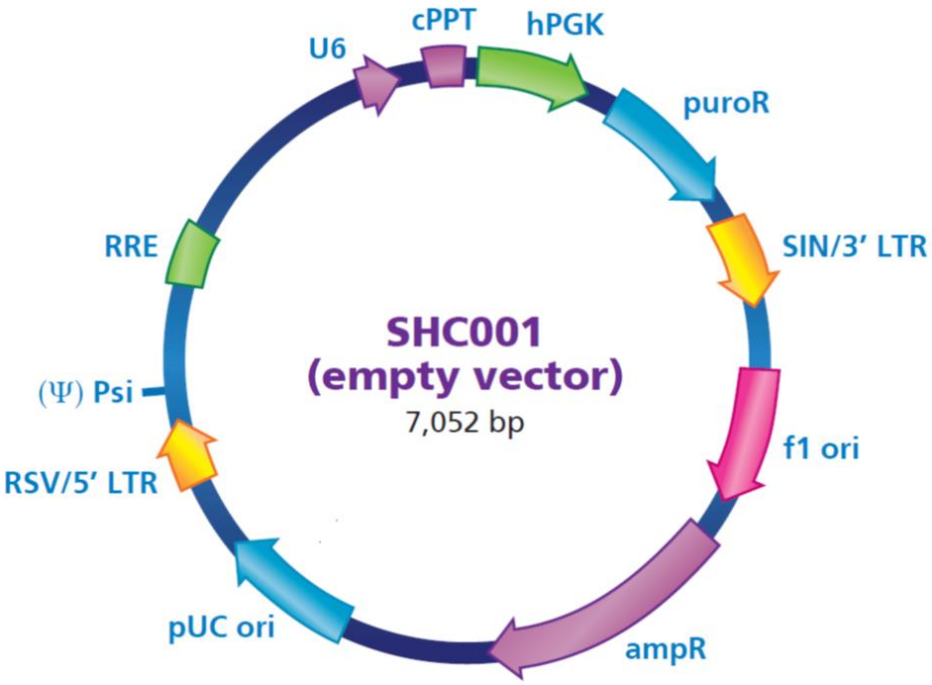
### 4.7.1. Generation of lentiviral particles.

For the production of lentiviral particles harboring silencing RNA, we transfected HEK 293T cells with plasmids encoding structural lentiviral proteins, control RNAs (or none) or silencing RNAs (**Figure 6**) (**Table 3**). HEK293T cells were cultured in plates starting at 1/10 dilutions until they reached 80 % confluence. Per 55-$cm^2$plate, 750 µl of Opti-MEM, 6 µg of packaging vectors (2 µg of pMD2.G, 2 µg of pMDLg/pRRE and 2 µg of pRSV-Rev), 8 µg of DNA of interest (pLKO.1, pLKO.1-shGFP, pLKO.1-shSec22b or pLKO.1-shTAPL) and 24 µl of PLUS reagent (which enhances transfection mediated by cationic lipids) were mixed. In parallel, 750 µl of Opti-MEM and 36 µl of lipofectamine per 55-$cm^2$ plate were mixed. For

further specifications of the transfected plasmids, see **Table 3** and **Figure 6**. After 5 min separate incubation at room temperature, all reagents were mixed and left 15 more min at room temperature.HEK293T cells were washed with PBS, and 3.5 mL of Opti-MEM plus 1.5 mL of transfection mix were added to the cells and cultured overnight at 37ºC. The following day cells were washed and fresh DMEM medium was added and cells were incubated for 24 h to produce and release lentiviral particles to the medium. Lentivirus-containing medium was then collected and filtered with 0.45 µm filters, aliquoted and frozen at -80ºC.

| | Plasmid | Gene expression | GFP expression | shRNA sequence | Reference |
|---|---|---|---|---|---|
| **Lentiviral packaging** | **pMD2-G** | Env | No | - | (Dull *et al.*, 1998) |
| | **pMDLg/pRRE** | gag | No | - | (Dull *et al.*, 1998) |
| | **pRSV-REV** | pol | No | - | (Dull *et al.*, 1998) |
| **Control plasmids** | **pLKO.1 (SHC001)** | - | Yes | - | (Moffat *et al.*, 2006) |
| | **pLKO.1-shGFP (SHC002)** | shRNA anti-GFP | No | CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT | (Moffat *et al.*, 2006) |
| **Silencing plasmids** | **pLKO.1-shTAPL** | shRNA anti-TAPL | Yes | CCGGCCGTGTATTGTTGGATGGCAACTCGAGTTGCCATCCAACAATACACGGTTTTTG | (Moffat *et al.*, 2006) |
| | **pLKO.1-shSec22b** | shRNA anti-Sec22b | No | CCGGCCCTATTCCTTCATCGAGTTTCTCGAGAAACTCGATGAAGGAATAGGGTTTTTG | (Moffat *et al.*, 2006) |

**Table 3. List of plasmids used in this work.**




| Description | Vector Element |
|---|---|
| **U6** | Human U6 promoter. It drives RNA Polymerase III transcription for generation of shRNA transcripts. |
| **cPPT** | Central polypurine tract. It improves transduction efficiency by facilitating nuclear import of the vector's preintegration complex in the transduced cells. |
| **hPGK** | Human phosphoglycerate kinase promoter. It grants constitutive expression of puroR. |
| **puroR** | Puromycin resistance gene for selection of pLKO.1 plasmid in mammalian cells. It encodes puromycin N-acetyl transferase, which acetylates puromycin, inactivating it. |
| **SIN/3'LTR** | 3' Self-inactivating long terminal repeat. |
| **f1 ori** | f1 bacterial origin of replication. |
| **ampR** | Ampicillin resistance gene for selection of pLKO.1 plasmid in bacterial cells. |
| **pUC ori** | pUC bacterial origin of replication. |
| **RSV/5'LTR** | 5' long terminal repeat. |
| **(Ψ) Psi** | RNA packaging signal. |
| **RRE** | Rev response element. |

**Figure 6. Map and elements description of the pLKO.1 negative control vector.**

All the plasmids based in pLKO.1 share this common structure. In the case of harboring a shRNA sequence, this is located right after the U6 promoter.

#### 4.7.2. Lentiviral transduction

##### 4.7.2.1.BMDC

Two hundred thousand BMDC, harvested 3 days after extraction of bone marrow cells, were cultured in flat-bottomed 96-well plates in 200 µl of IMDM medium supplemented with 20 ng/mL of GM-CSF. Three days later, plates were centrifuged for 2 min at 2200 r.p.m. and the medium was collected and stored. Fifteen microliters of lentiviral particles were added to each well together with 35 µl of IMDM medium supplemented with polybrene at a final concentration of 8 µl/mL. Plates were then centrifuged at 37ºC at 2200 r.p.m. for 90 min. Lentiviral particles were removed and 100 µl of previously stored conditioned IMDM medium was added to the wells together with 100 µl of fresh IMDM medium. Three days later positive selection was carried out by adding puromycin 5 µg/mL for 2 days.

##### 4.7.2.2. RMA and RMA-S

One hundred thousand cells were cultured in flat-bottomed96-well plates in 200 µl of DMEM medium. Plates were centrifuged at 2200 r.p.m. for 2 min and medium was discarded. Ten microliters of lentiviral particles together with 10 µl of fresh DMEM medium supplemented with polybrene at a final concentration of 8 µl/mL were added to each well. Plates were centrifuged at 37 ºC at 1200 r.p.m. for 45 min, then 80 µl of medium was added and cells were incubated for 6 h at 37ºC. Cells were again centrifuged and lentivirus-containing medium was replaced by 100 µl of fresh medium. Two days later positive selection was carried out.

The differences in the trandsduction protocol between BMDC and RMA or RMA-S are due to the difference growth rate (fewer RMA or RMA-S cells are cultured in the beginning of the protocol), the need of maturation factors for BMDC (three-days enriched medium is not discarded for BMDC) and the difference in lentiviral transduction success (smaller volume quantities of lentiviral particles are used for RMA or RMA-S cells and the second centrifugation does not need to be as long as it does for BMDC).

### 4.8. Western blotting

#### 4.8.1. Protein extraction.

One million BMDC or RMA cells were pelleted by centrifugation and lysed in radio immunoprecipitation assay buffer (RIPA: 150 mM sodium chloride, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 50 mM Tris-HCl pH 8.0) with Complete

protease inhibitors (Roche) preheated at 95 ºC. Cells were lysed at 95 ºC for 15 min and DNA was discarded by centrifuging the lysate at 15000 r.p.m. for 15 min. at R.T. The supernatant was collected and protein was quantified using the BioRad Bradford Protein Assay kit. Sample buffer (60 mM Tris-HCl pH 6.8, 10 % glycerol, 1.25 % β-mercaptoethanol, 0.01 % bromophenol blue (w/v), 2 % SDS) was added to each sample in a dilution 1/6 (sample buffer/sample) and samples were boiled at 95 ºC for 5 min to denature the proteins. Samples were stored at -20 ºC.

#### 4.8.2. Immunoblotting.

Thirty µg of protein samples were loaded onto10% acrylamide SDS-PAGE gels, and proteins were transferred for 2.5 h at 4 ºC to nitrocellulose membranes (Hybond-ECL, GE Healthcare) in a tank system (BioRad). Membranes were blocked in a 5 % powder milk in PBS-Tween 0.1 % solution overnight at 4 ºC, washed and incubated with primary antibodies for 1 h at 4 ºC. Primary antibodies used for immunoblotting are summarized in the Antibodies section 4.1.3. After washing the primary antibody with PBS-Tween 0.1 % three times, the membranes were incubated with the secondary antibody for 1 h at 4 ºC. The membranes were developed by chemiluminescence (ECL, GE Healthcare).

## 4.9. RNA quantification.

#### 4.9.1. RNA extraction.

RNA samples were extracted from RMA and RMA-S cells with TRIzol Reagent (Life Techonologies). Briefly, cells were lysed in TRIzol Reagent, mixed with chloroform and centrifuged. The aqueous phase containing the RNA was collected and placed in a new tube. Isopropanol was added and the sample was centrifuged again to precipitate the RNA. After precipitation, the RNA pellet was washed with 75 % ethanol and air dried. The RNA was resuspended in RNase-free water, heated at 55 ºC for 10 min, and stored at -80 ºC. RNA purity and concentration were determined by using a Nanodrop ND-1000 (Thermo Scientific). RNA integrity and DNA contamination were determined by agarose gels and a Bioanalyzer Agilent 2100 (Agilent).

#### 4.9.2. Quantitative reverse transcription PCR (RT-qPCR).

RT-qPCR was performed in intron-spanning assays using the primers listed in **Table 4** by the Genomics Facility of the Centro de Biología Molecular Severo Ochoa. Primers were designed using the software ProbeFinder and primer specificity was checked by performing BLAST using the NCBI tool Primer-BLAST.

| | Gene | | Primers | |
|---|---|---|---|---|
| | | | **Forward** | **Reverse** |
| **Normalizer genes** | **β2m** | | TACATACGCCTGCAGAGTTAAGCA | TGATCACATGTCTCGATCCCAG |
| | **Dgcr8** | | GGGGTTCCTTACTACGCATGT | CACACTCTTGTCAGAGGTCTCCT |
| | **Gapdh** | | CACCACCAACTGCTTAGCCC | TGTGGTCATGAGCCCTTCC |
| | **Map2k2** | | CAGACTTCCAGGAGTTTGTGAAT | CTTGATGAAGGCGTGGTTC |
| | **Tap1** | | GTGCACAGAGAGGTGTTTCG | CAGTCACCCGAGATGTGATG |
| | **Tbp** | | CCACAGGGCGCCATGA | GCTGTGGAGTAAGTCCTGTGCC |
| | **Ywhaz** | | TTACTTGGCCGAGGTTGCT | TGCTGTGACTGGTCCACAAT |
| **Genes of interest** | **Sec22b** | Design 1 | AGAAGAAAGGCCCAGCTTAAA | TTGGGAACGCATAGACAAAGT |
| | | Design 2 | TGGTGGCCAATATTGAAGAAG | TCAAAGGCTAACAATTTGTCCA |
| | | Design 3 | TGGTTTTGTGTGAAGCTGCC | GCATTGGATTCAAAGGCTAACAA |
| | **Abcb9 (TAPL)** | Design 1 | CCGTGTATTGTTGGATGG | AGATGTTGTCTGTGATGGA |
| | | Design 2 | CCCACACACAGGTCCTACAG | CGTGTGTGAACATCCTGGA |
| | | Design 3 | CCTGGAGAACTTCTACCCTCTGC | GGCGCCTATGACCACAAGTAC |

**Table 4. Primers used for RT-qPCR.**

Three different primer designs were developed for genes Sec22b and TAPL to avoid false positive results due to the location of the primers (Shepard *et al.*, 2005). For both genes we designed three intron-spanning and non intro-spanning pairs of primers in different regions of the genes (**Figure 7**).

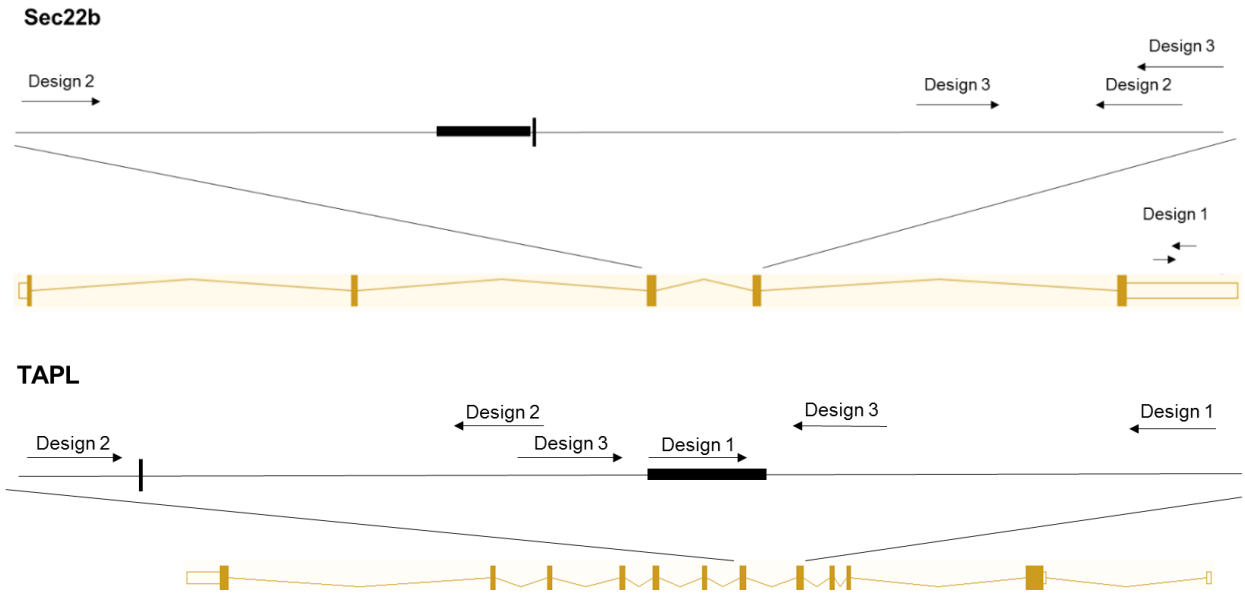



**Figure 7. Scheme of the position of the primers used in the RT-qPCR.**

The maps of the genes Sec22b and TAPL are extracted from Ensembl. Black thick lines indicate the location of the interfered zone. Designs of primers to amplify three different gene regions of Sec22b or TAPL are indicated by arrows. Vertical lines indicate the position of introns.

The retrotranscriptase reactions were performed manually using the retrotranscription kit iScript cDNA Synthesis kit (BioRad, 170-8891) containing random primers and oligo-dT, following manufacturer's instructions. For the reaction, 1 µg of RNA in a final volume of 20 µl was used.

Amplification was performed using the master mix Power Sybr Green (Applied Biosystems) following the amplification protocol: 10´ at 95ºC + (15" at 95ºC + 1´ at 60ºC) x 40 + 5'' at 60ºC + 5'' at 95ºC. Fluorescence acquisition was performed at the end of the elongation step (1' at 60 ºC) during the amplification and during all the temperature slope in the melting cycle (5'' at 60 ºC). The specificity of the amplification product was checked by monitoring a denaturing cycle (from 60 to 95 ºC) on the final amplified product. Only one product was observed for all the genes analyzed, verifying the specificity of the amplification product.

Data were processed using the software GenEx v5.4.4, which performs the following processing steps: 1. Primers efficiency correction; 2. Calculation of the mean of triplicates of the qPCR; 3. Normalization of the data ($2^{-\Delta Ct}$) (Ct, "threshold cycle": the cycle number at which the fluorescence signal crosses the threshold at which it occurs significant and specific amplification), using as a reference the expression of the gene Map2k2 (established as one of the most stable genes included in the experiment by the software NormFinder), correcting the variability between experiments and 4. Relative quantification of each gene in the sample of interest (pLKO.1-shSec22b and pLKO.1-shTAPL) in relation to the expression of the same gene in the calibrating sample (pLKO.1 or pLKO.1-shGFP) ($2^{-\Delta\Delta Ct}$) when we analyzed silencing of genes Sec22b and TAPL after transduction with lentiviral particles.

## 4.10. Statistical analyses.

Analyses of the data are based in mean values and errors expressed as standard deviation (SD) for RT-qPCR data or standard error of the mean (SEM) for the rest of the experiments. To compare means between different groups of data, unpaired two-tailed t tests were performed. In the case of the experiments measuring viral titer, Mann-Whitney nonparametric t-tests were performed due to a non-Gaussian distribution of the data. This test compares differences between medians. The significance of the difference was given by the p value (***, p value ≤ 0.001; **, p value ≤ 0.01; *, p value ≤ 0.05; non-significant, p value > 0.05). The software used to perform statistical analyses was Graph Pad Prism 5.

# 5. RESULTS.

## 5.1. Involvement of transporters and vesicular transport involved in TAP-independent antigen presentation.

The ATP-binding cassette (ABC) family is a family of transporters that includes TAP that use the energy of ATP to transport a vast diversity of molecules (Theodoulou and Kerr, 2015; Zolnerciks *et al.*, 2011; Hinz and Tampe, 2012). Cells count on many transporters within the ABC family, each of them with different transport functions (Hickman *et al.*, 2013; Sasaki *et al.*, 2011; Looker *et al.*, 2015a; Zhao *et al.*, 2006). One possible explanation to TAP-independent antigen processing is that it may exist another peptide transporter that could transport some MHC-I ligands or precursors to MHC-I compartments, and therefore bypass TAP. We proposed one of the members of the TAP family, Abcb9 (also known as TAP-like or TAPL), as a candidate alternative transporter of MHC-I binding peptides, since it is known to participate in the transport of MHC-II-binding peptides (Zhao *et al.*, 2006).

We also aimed to know more about the vesicles involved in this kind of alternative routes using the SNARE proteins as a tool to study vesicle trafficking. The SNARE Sec22b protein is expressed in the ER membrane and it is involved in ER to Golgi transport (Gordon *et al.*, 2010)as well as in the fusion of ER-derived vesicles to phagosomes(Becker *et al.*, 2005).It has been implicated in TAP-dependent antigen cross-presentation (Cebrian *et al.*, 2011). Therefore, assessing whether TAP-independent cross-presentation is dependent on Sec22b or not, would tell us if TAP-independent cross-presentation routes depend on the ER and ER-derived compartments or not.

To study the possible involvement of TAPL and Sec22b in TAP-independent viral antigen processing, we generated lentiviral particles harboring plasmids with shRNA sequences to silence the expression of either the transporter TAPL, the SNARE protein Sec22b or none of them as a negative control.

### 5.1.1. Effect of Sec22b silencing on cross-presentation of OVA-coated beads.

To study the involvement of the SNARE protein Sec22b on TAP-independent cross-presentation by BMDC, we generated lentiviral particles harboring either an RNA complementary to the Sec22b transcript (shSec22) or a RNA anti GFP as a negative control. We transduced C57BL/6 and TAP1$^{-/-}$ BMDC with the resulting lentiviral particles and checked the effect of shSec22b RNA on the expression of Sec22b protein and the results show a specific decrease in Sec22b protein levels in shSec22b-transduced BMDC (**Figure 8**).

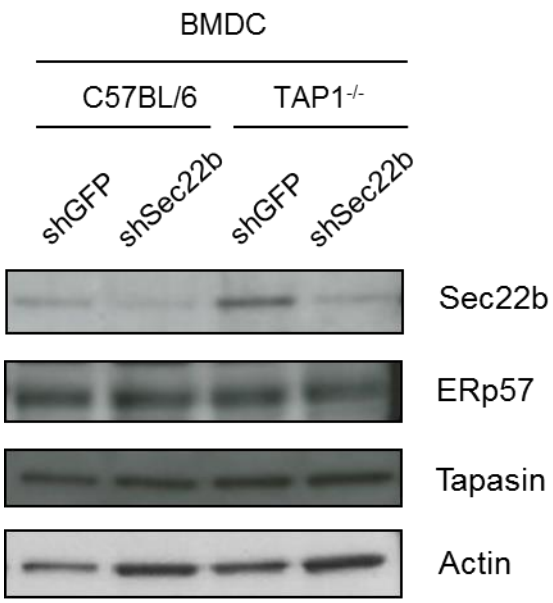



**Figure 8. Sec22b protein levels decrease after specific shRNA lentiviral transduction in BMDC.**

C57BL/6 and TAP1$^{-/-}$ BMDC were transduced with lentiviral particles harboring either antiGFP RNA or shSec22b RNA. Five days later, immunoblotting with Sec22b, ERp57, Tapasin and Actin antibodies was performed.

It was already described that cross-presentation of OVA from OVA-coated beads by C57BL/6 BMDC was dependent on Sec22b, possibly due to its role in ER-derived vesicle fusion to phagosomes (Cebrian *et al.*, 2011). We wondered if the previously described OVA-coated beads proteasome-dependent and TAP-independent cross-presentation (Merzougui *et al.*, 2011) also needed Sec22b-dependent PLC supply from the ER to the phagosomes and therefore, if it was Sec22b dependent. If TAP-independent epitopes access the ER or ER-derived compartments, TAP-independent cross-presentation would need Sec22b function. On the other hand, Sec22b-independency would mean either that the presentation of the epitope does not require the components of the ER or that there is some undescribed Sec22b-independent anterograde transport from the ER.

The results reproduce the previously described Sec22b dependency on cross-presentation by C57BL/6 BMDC but they show no need of this protein for cross-presentation of OVA-coated beads byTAP1$^{-/-}$ BMDC (**Figure 9**).

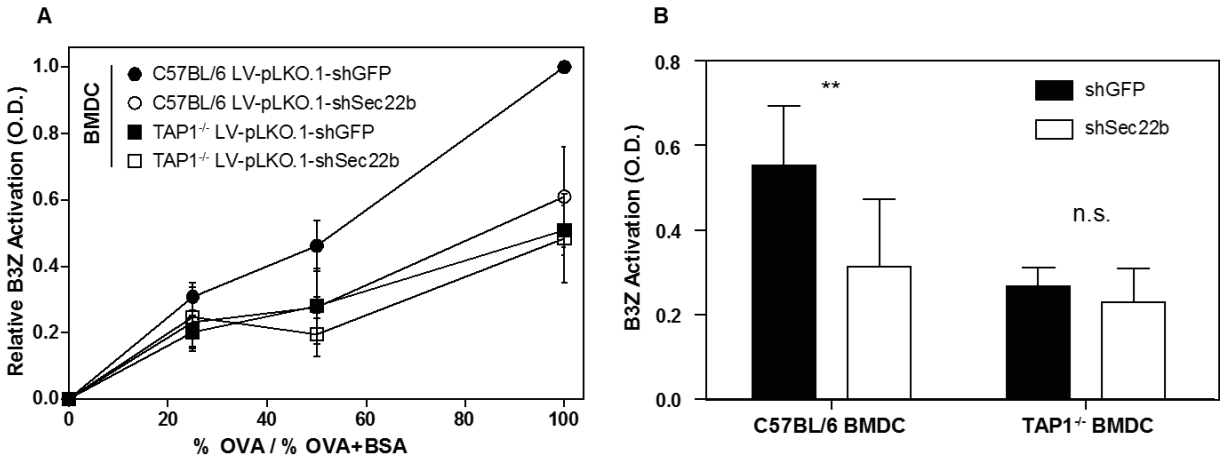



**Figure 9. OVA-coated beads cross-presentation is Sec22b-dependent in C57BL/6 BMDC but Sec22b-independent in TAP1$^{-/-}$ BMDC.**

C57BL/6 and TAP1$^{-/-}$BMDC were transduced with lentiviral particles harboring an antiGFP RNA or a shSec22b RNA. The effect of silencing Sec22b on OVA cross-presentation to B3Z T cells was analyzed by incubating BMDC with 3 µm beads coated with different quantities of the antigen and the protein BSA as a coating control. After incubation with the beads, BMDC were co-cultured with B3Z T-cell hybridoma. T cell activation was measured as LacZ activity of B3Z T-cell hybridoma using a spectrophotometer and data were relativized to the highest value of activation in each experiment. Mean activation values and SD values for three independent experiments are shown (**A**). Data corresponding to beads loaded only with OVA from three previous experiments were statistically analyzed by a two-tailed t-test (**B**).

### 5.1.2. Use of lentiviral vectors to silence Sec22b and TAPL gene expression.

After knowing the lack of involvement of Sec22b on TAP-independent OVA cross-presentation by BMDC, we wanted to know if transport mediated by Sec22b was participating in TAP-independent direct presentation of viral antigens. Also, we wanted to assess the possible role of the transporter TAPL in this kind of direct antigen presentation. We used lentiviral particles in the attempt to silence these genes separately. We changed the negative control plasmid from pLKO.1-shGFP to pLKO.1 expressing GFP and transfected HEK 293T cells with lentiviral packaging plasmids and pLKO.1 and pLKO.1-shTAPL, both GFP and puromycin resistance expressing plasmids (**Table 3**).We confirmed the success of the transfection by GFP expression (**Figure 10**). The resulting lentiviral particles were collected and used to transduce C57BL/6 and TAP1$^{-/-}$ (BMDC) and lymphoma-derived RMA or RMA-S cells. RMA-S cells area mutant cell line derived from RMA that lack the expression of TAP2 (Ljunggren *et al.*, 1991; Zhou *et al.*, 1993). To select the successfully transduced cells, we added puromycin to the cell culture and percentage of living cells and GFP expression were tracked over time (**Figure 11**).

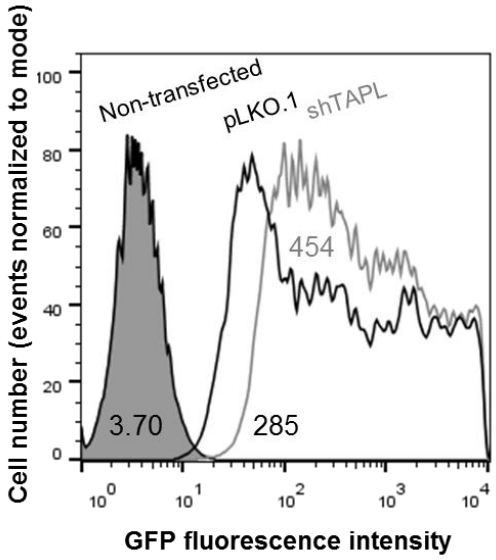



**Figure 10. HEK293T cells are efficiently transfected.**

HEK293T cells were transfected with plasmids pLKO.1 (black empty histogram), pLKO.1-shTAPL (grey empty histogram) or non-transfected (black filled histogram). Forty-eight hours after transfection, GFP expression in HEK293T cells was measured by flow cytometry. Numbers show the values of mean GPF fluorescence intensity.

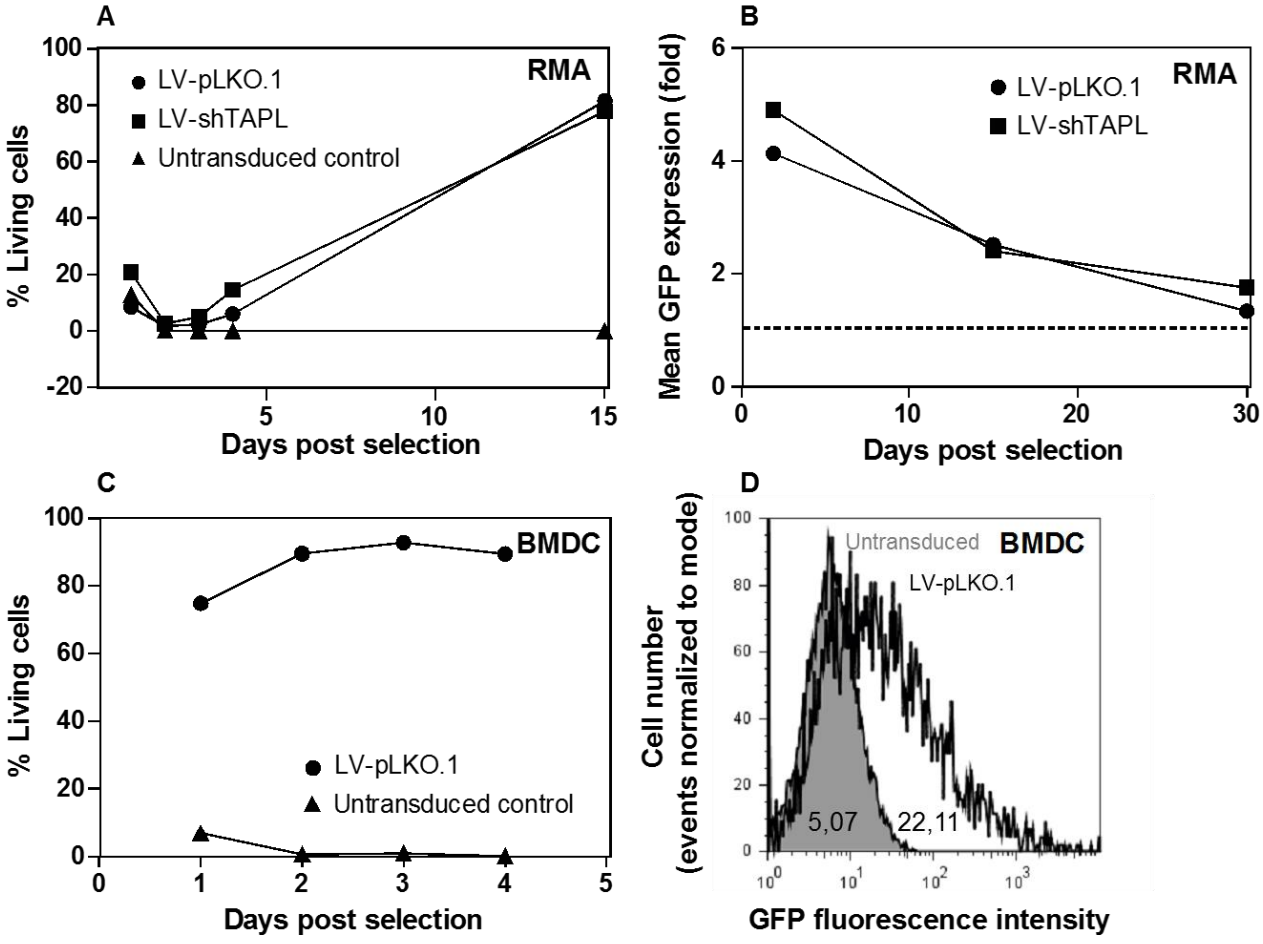



**Figure 11. Cell survival and GFP expression after lentiviral transduction of RMA cells and BMDC.**

RMA cells and BMDC were transduced with lentiviral particles harboring a shTAPL RNA (shTAPL) or only the vehicle plasmid (pLKO.1), both expressing GFP. Three days later, puromycin was added for selection of transduced cells. The percentage of living cells (**A**, **C**) and GFP expression (**B**, **D**) of RMA cells (A, B) and BMDC (C, D) was measured by flow cytometry at different days. The kinetics of GFP expression is calculated as the fold increase of the mean fluorescence of transduced cells to the untransduced control (dotted line) for RMA (**B**) and shown at five days for BMDC (untransduced control, filled histogram; LV-pLKO.1, empty histogram) (**D**).

Although the efficiency of transduction was low for lymphoma cells, we chose these as a model to perform our experiments. First, we had a short time frame for testing with CTL since BMDC greatly lose their capacity to be infected by VACV after ten days in culture (transduction and selection protocol alone takes eight days). Second, there was a lack of reproducibility in the lentiviral transduction of BMDC. Third, sensitive and specific CTL lines were frequently unavailable when needed.

To establish non-transient transduced lymphoma cell lines (that did not lose infectivity over time) we had to wait for more than 30 days to have enough control, shTAPL and shSec22b in both RMA and RMA-S cells to be able to check gene expression and perform further experiments. As we did not succeed in stablishing a RMA cell line transduced with control plasmid pLKO.1-shGFP, we used pLKO.1 which did not express any shRNA (**Table 3**) as a negative control for experiments with RMA cells.

To check if the expression of the proteins of interest was reduced, we performed immunoblotting of RMA cells with confusing results, since we obtained two different bands when detecting TAPL, one with the expected molecular weight (75 KDa) and one with a non-expected weight (50 KDa). The expected band did not decrease in shTAPL-transduced cells while the non-expected band did decrease in the same cells (**Figure 12**). Immunoblotting of Sec22b in RMA and RMA-S cells resulted in several bands around the expected molecular weight and none of them decreasing after shSec22 transduction (data not shown).

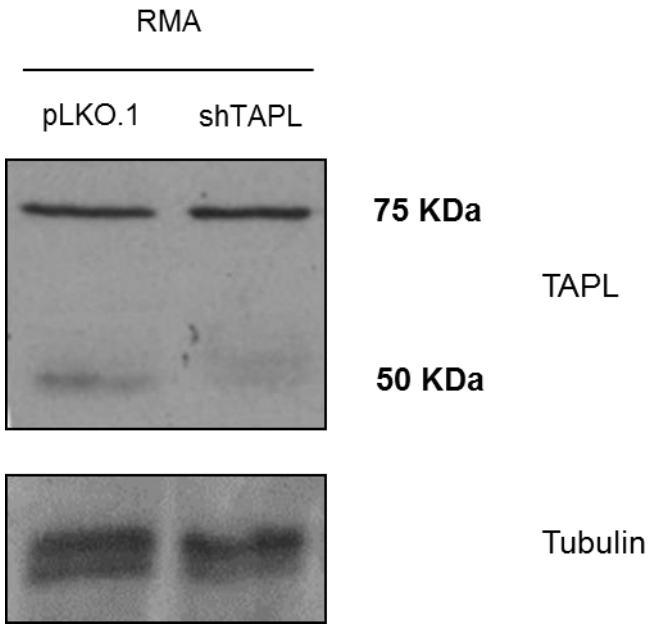



**Figure 12. Anti-TAPL immunoblotting after shTAPL lentiviral transduction of RMA cells.**

Five weeks after lentiviral transduction, proteins were extracted from RMA cells and an immunoblotting to detect TAPL was performed. Tubulin was immunoblotted as a loading control.

We tried another method to measure gene expression. RT-qPCR of two individual samples of the same cells was performed to measure the expression of TAPL and Sec22b, with three different pairs of primers for each gene. Also, to have several candidates to normalize the data, primers for Ywhaz, Gapdh, Tap1, Dgcr8, β2m, Tbp and Map2k2 genes were also included in the experiment. After the experiments, we chose Map2k2 as the best candidate for normalization due to its highly stable expression shown. The RQ values (RQ = relative quantification = $2^{-\Delta\Delta Ct}$) show no inhibition of the expression of any of the genes neither in RMA nor in RMA-S cells (**Figure 13**).These results agree with the expression of GFP in transduced cells, which is lost over time. On the other hand, puromycin resistance is not lost in transduced cells, probably due to a selective pressure that did not affect the expression of GFP. We think that the unlikely dissociation between GFP expression and puromycin resistance happened because of the survival of cells after eventual loss of GFP expression, while eventual loss of puromycin resistance expression would lead to cell death in the presence of puromycin.

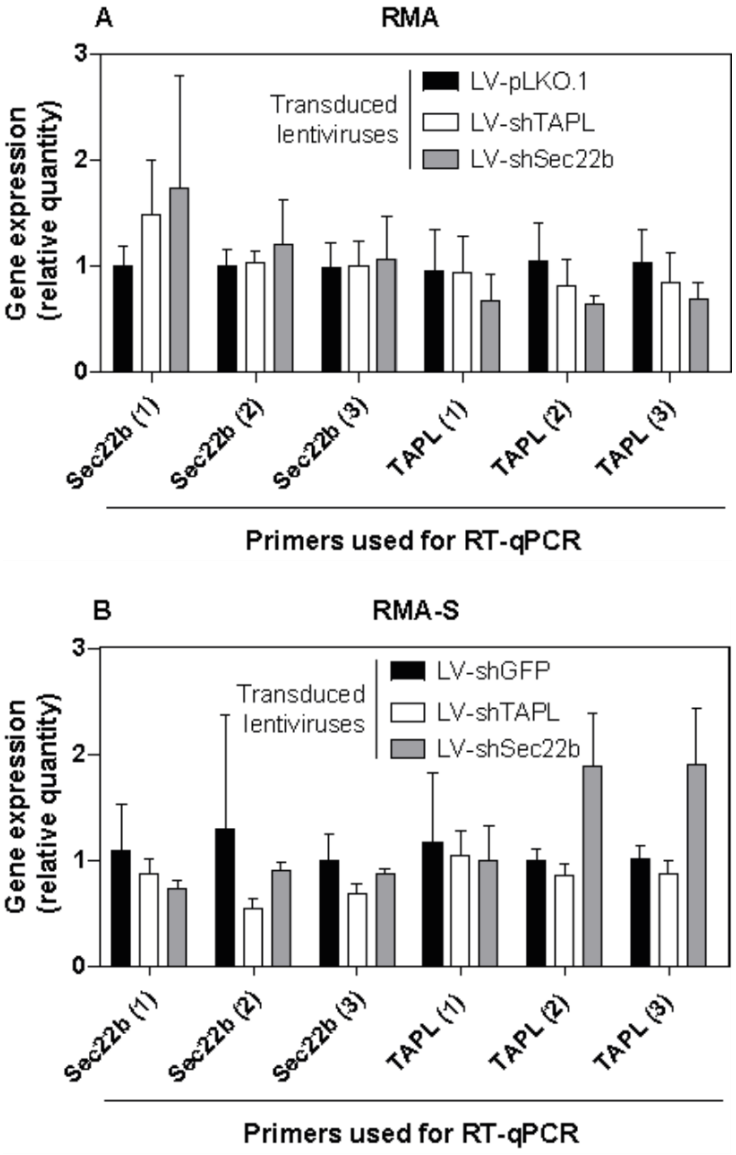



**Figure 13. Detection of the expression of Sec22b and TAPL genes in RMA and RMA-S cells by RT-qPCR after lentiviral transduction.**

Two individual RNA samples from RMA (**A**) and RMA-S (**B**) cells transduced with each of the three indicated lentiviruses were used to measure the expression of the genes Sec22b and TAPL by RT-qPCR using three different pairs of primers for each of them. Gene expression is relativized using the ΔΔCt method (Livak and Schmittgen, 2001). Mean and SD values are shown. Triplicates of the RT-qPCR for each sample were performed.

Since we failed in silencing any of the genes in RMA and RMA-S cells, we could not perform further experiments to study the possible involvement of TAPL and Sec22b in TAP-independent direct antigen presentation.

We analyzed the data corresponding to the cells transduced with no shRNA to obtain a side result checking if the expression of all the genes included in the RT-qPCR was relevantly different in RMA compared with RMA-S cells. The results show a very similar profile for the two kind of cells except for the transporters TAPL and TAP1and the gene Dgcr8, involved in the biogenesis of microRNA, which was higher in RMA cells (2.46-fold± 0.58 for TAPL and 2.67-fold± 0.25 for TAP1) (**Figure 14**).

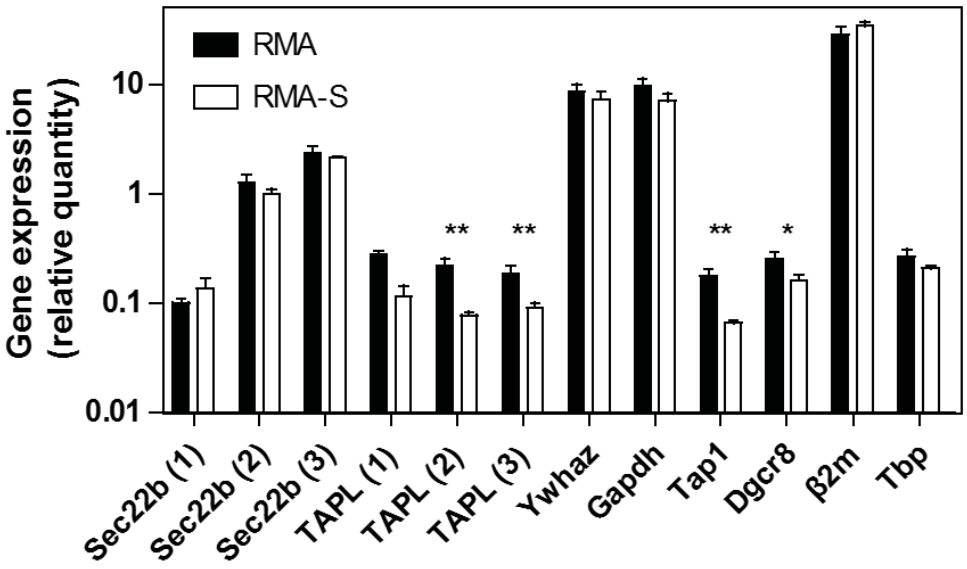



**Figure 14. Comparison of RMA and RMA-S gene expression.**

RNA samples were extracted from RMA and RMA-S cells transduced with lentiviral particles with no shRNA to perform RT-qPCR for several genes (Ywhaz: Tryptophan 5-Monooxygenase Activation Protein; Gapdh: Glyceraldehyde-3-Phosphate Dehydrogenase; Tap1: Transporter associated with antigen processing 1; Dgcr8: DiGeorge Syndrome Critical Region Gene 8; β2m: beta 2 microglobulin; Tbp: TATA Box Binding Protein). For the genes Sec22b and TAPL, three different pairs of primers were designed and included in the experiment. Two individual RNA samples for each cell type were included and triplicates for each sample were performed. The expression of the gene Map2k2 was used as a normalizer and the relative quantity mean and SD values of the indicated genes are shown.

Finally, we aimed to check if TAP-independent viral antigen presentation routes are equally efficient in our two models of presenting cells, primary BMDC and the lymphoma cell line RMA. We performed ICS assays using purified $CD8^+$ T lymphocytes from VACV-infected C57BL/6 mice as effector cells and either VACV-infected C57BL/6 BMDC or VACV-infected

RMA cells as antigen presenting cells. TAP-deficient counterparts TAP1$^{-/-}$ BMDC and TAP2$^{-/-}$ RMA-S cells were included in the experiment, and the CD8$^{+}$ T lymphocyte activation was measured. The efficiency of TAP-independent routes in both models was calculated by dividing the CD8$^{+}$ activation after the stimulation with infected TAP-deficient cells by the activation after the stimulation with infected TAP-proficient cells. The results show a greater use of TAP-independent routes in BMDC than in the lymphoma cell lines (**Figure 15**).

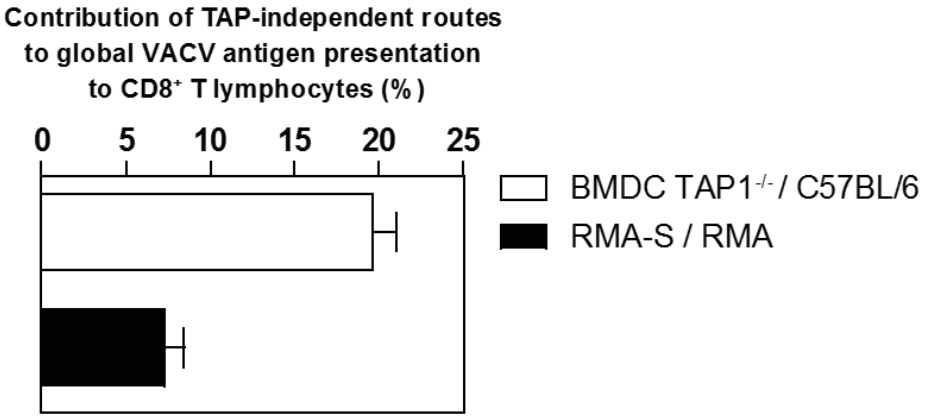



**Figure 15. TAP-independent routes are proportionally less efficient in the TAP2$^{-/-}$ lymphoma cell line RMA-S than in TAP1$^{-/-}$ BMDC.**

CD8$^{+}$ T lymphocytes from VACV WR-infected C57BL/6 mice were purified 6 d.p.i. and stimulated in culture either with VACV WR-infected TAP1-proficient or TAP1-deficient BMDC, or VACV WR-infected TAP2-proficient (RMA) or TAP2-deficient (RMA-S) lymphoma cells. IFNγ production by CD8$^{+}$ T lymphocytes was measured by flow cytometry and the percentage of activation values against TAP-deficient/TAP-proficient cells was calculated separately for BMDC and for the lymphoma cell lines following the formula: (T-lymphocyte activation against TAP-deficient cells/T-lymphocyte activation against TAP-proficient cells)x100. Mean values and SEM of 2 to 4 effector:target ratios are shown.

## 5.2. Proteolytic enzymes involved in TAP-independent antigen processing.

### 5.2.1. Contribution of proteasomes to the generation of TAP-dependent and TAP-independent epitopes.

Proteasomes are known to be the proteases with major proteolytic activity in the cell (Kloetzel and Ossendorp, 2004; Shastri *et al.*, 2005) and, working together with other proteases in the cell, they generate many epitope precursors (Lázaro *et al.*, 2015). For years, it has been believed that TAP-ind epitopes do not require proteasomal function since it is assumed that TAP-ind epitopes are not generated in the cytosol, and that they bypass TAP transport by the parental proteins using the Sec61 translocon or by other unknown

mechanisms. In fact, many engineered proteins provide TAP-ind epitopes in this way, not requiring any cytosolic machinery(Medina *et al.*, 2009; Kim *et al.*, 2011; Elliott *et al.*, 1995; Snyder *et al.*, 1994; Bacik *et al.*, 1994; Raafat *et al.*, 2011). However, in the last years, it has been known that proteasomes are involved in TAP-ind antigen processing (Oliveira *et al.*, 2014). With the aim of characterizing and quantifying the role of proteasomes in alternative TAP-ind antigen processing pathways acting on natural VACV proteins, we performed ICS assays with $CD8^+$ T lymphocytes purified from spleens extracted from C57BL/6 VACV-infected mice. We stimulated the effector cells with VACV-infected C57BL/6 or $TAP1^{-/-}$BMDC treated or not with the proteasome inhibitor lactacystin (LC) and measured the $CD8^+$ response (**Figure 16**). The results show that in both C57BL/6 and $TAP1^{-/-}$ infected BMDC the treatment with LC impairs viral antigen presentation.

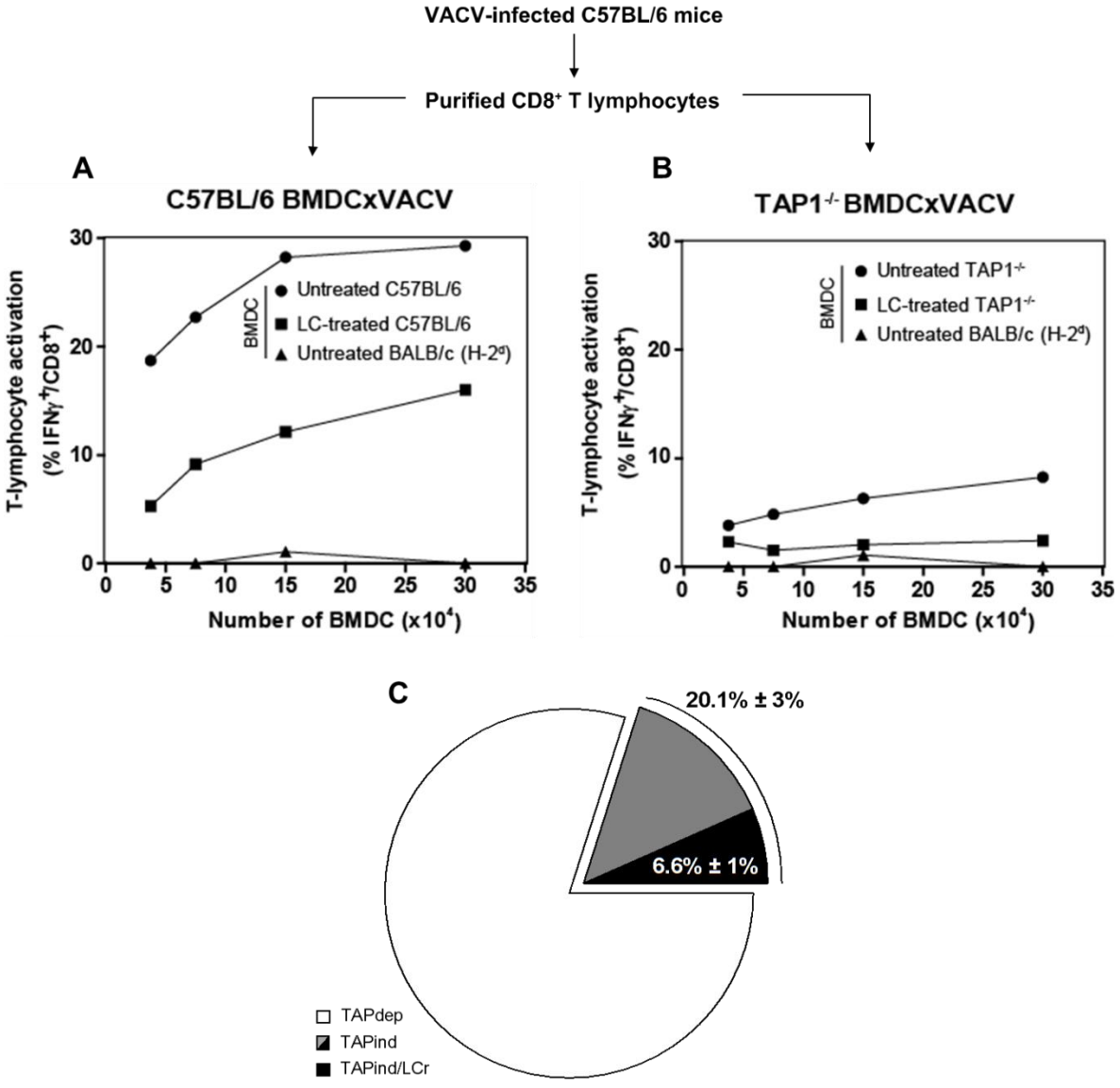



**Figure 16. The proteasome inhibitor lactacystin partially impairs antigen presentation in both C57BL/6 and $TAP1^{-/-}$ BMDC.**

Purified $CD8^+$ T lymphocytes from spleens extracted from VACV WR-infected C57BL/6 mice five days post-infection were stimulated for 6 hours with VACV WR-infected C57BL/6 (**A**) or $TAP1^{-/-}$ (**B**) BMDC treated or not with LC 10 µM during infection. In all cases $CD8^+$ T lymphocytes were also stimulated in parallel with VACV WR-infected BMDC harboring a negative control haplotype ($H\text{-}2^d$). One representative experiment out of four is shown. Relative contribution to $CD8^+$ T-lymphocyte activation of alternative antigen processing pathways (TAP-independent and TAP-independent/LC-resistant) was calculated in non-saturating effector:target ratios using data from four experiments. Mean values and SD are shown (**C**).

To compare the role of proteasomes in both global (TAP-dependent and TAP-independent together) and TAP-independent antigen presentation alone, we quantified the results from **Figure 16A** and **Figure 16B** by dividing the $CD8^+$ T lymphocyte activation after LC-treatment by the activation against non-treated VACV-infected cells in both C57BL/6 and $TAP1^{-/-}$cells (**Figure 17**). In both genotypes, the percentage of remaining activation after inhibition of proteasomal activity is similar. Regardless of the possible incomplete inhibition of the proteasomes by the LC treatment (Garcia-Medel *et al.*, 2011), this would confirm that proteasomes play a very similar role in TAP-independent and TAP-dependent antigen presentation.

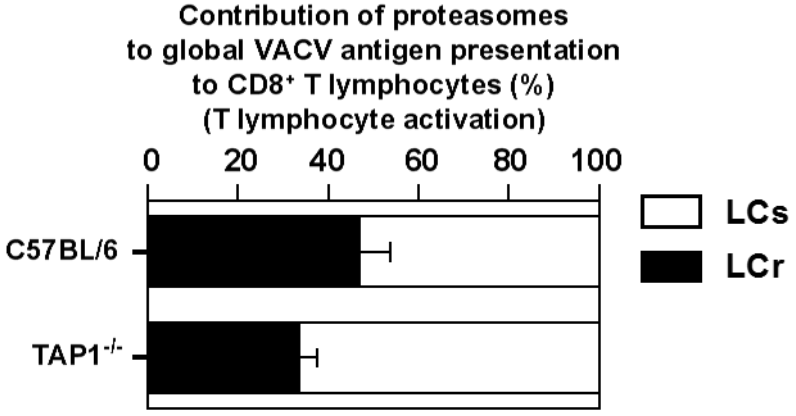



**Figure 17. Proteasomes contribute to antigen processing to the same extent in VACV WR-infected C57BL/6 and $TAP1^{-/-}$ BMDC.**

Purified $CD8^+$ T lymphocytes from spleens extracted from VACV WR-infected C57BL/6 mice five days post-infection were stimulated for 6 hours with VACV WR-infected C57BL/6 or $TAP1^{-/-}$BMDC treated or not with LC 10 µM during infection. The percentage of remaining antigen presentation in four experiments after LC treatment was calculated following the formula: (T-lymphocyte activation against LC-treated VACV WR-BMDC / T-lymphocyte activation against non-treated VACV WR-BMDC) x100for both C57BL/6 and $TAP1^{-/-}$ VACV WR-infected BMDC. Mean values and SEM of four independent experiments are represented.

Similar experiments with RMA and RMA-S cells were not performed due to the low level of TAP-independent antigen presentation.

### 5.2.2. Identification of TAP-independent and lactacystin-resistant viral antigen presentation routes by using viral antigen-specific CTL lines.

The *ex vivo* experiments showed $CD8^+$ activation after the stimulation with LC-treated VACV-infected $TAP1^{-/-}$ BMDC (**Figure 16B**). We wondered whether that occurred because LC did not completely block proteasomal function and therefore we might be detecting antigens generated by residual proteasomes or, on the other hand, there were indeed TAP-ind antigens that did not require proteasomal cleavage. To address these two possibilities, we studied the effect of LC on the generation of individual epitopes, rather than studying the collective presentation of all epitopes generated during a VACV infection. Previous results in our lab indicated that there were individual epitopes that resisted the treatment with LC, but all of them needed TAP to be presented (see **Table 5**later on) and therefore, they could not be responsible for the remaining presentation after LC treatment of infected $TAP1^{-/-}$ BMDC. For some VACV TAP-independent epitopes, the effect of LC had not been studied, because they were derived from post-replicative expression viral genes. Indeed, the treatment with proteasome inhibitors has the side effect that it impairs viral DNA replication, not allowing post-replicative viral proteins to be synthetized (Satheshkumar *et al.*, 2009) (**Figure 18A**). This added difficulty to our work that needed to be bypassed, so we designed a different approach to the antigen presentation assay by adding the proteasome inhibitor LC at different times post-infection. This allows the antigenic proteins to be synthetized first, which let us check the effect of LC treatment on the generation of the epitope rather than on the replication of viral DNA (**Figure 18B**).

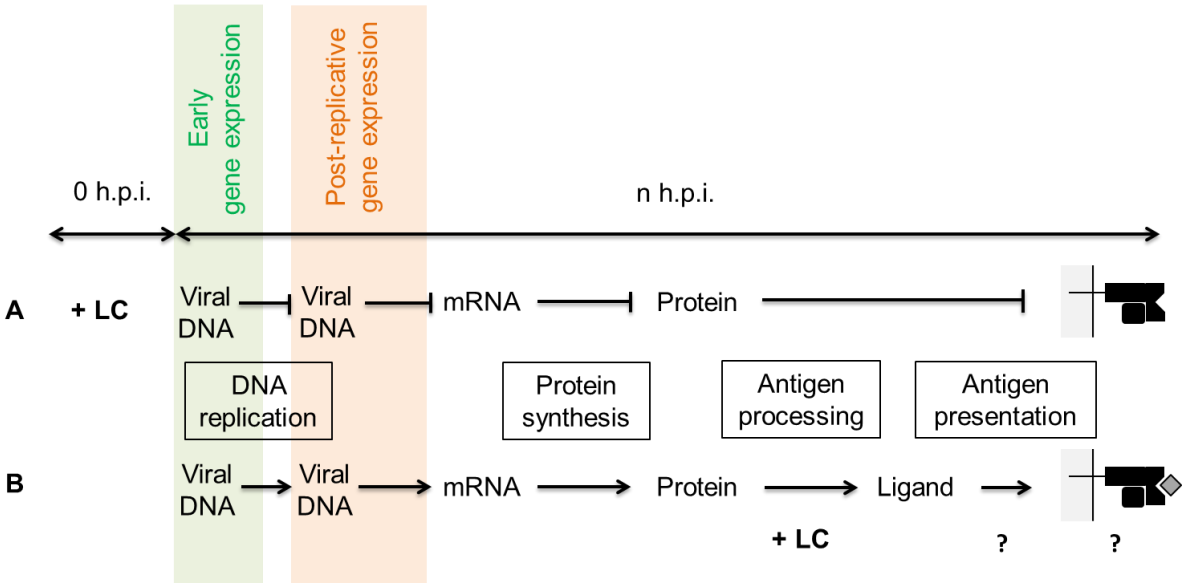


**Figure 18. Diagram showing the side effect of proteasome inhibitors on VACV-infected cells.**

LC treatment impairs viral DNA replication and therefore, expression of post-replicative genes and protein products and epitopes (**A**). Allowing the infected cell to synthesize viral proteins and adding the proteasome inhibitor LC at later times post infection would allow to solely assess the effect of the inhibition on the generation of the epitopes (**B**).

We generated CTL lines specific for TAP-ind antigens expressed by post-replicative expression genes. To generate CTL lines, we infected with VACV C57BL/6 mice, harvested their spleens and stimulated the splenocytes with the synthetic peptide of interest. With a total of six CTL lines we tested the involvement of proteasomes in antigen processing of each individual epitope. To this end, we performed $CD8^+$ T lymphocyte activation assays with VACV-infected C57BL/6 BMDC adding LC at different times during the infection. We also tested at different times after infection to make sure we could detect each antigen in non-saturating conditions. Since we did not have a quantifiable manner to classify the six epitopes as LC-enhanced ($LC^E$), LC-resistant ($LC^R$) or LC-sensitive ($LC^S$), we generated other two CTL lines specific for two epitopes derived from early expression genes, B8R which is known to be $LC^S$, and B2R, known to be $LC^E$ (Silvia Lázaro, personal communication). The addition of LC at different times post-infection blocked the presentation of B8R, but not the presentation of B2R, reproducing the previously known result (B8R as $LC^S$ and B2R as $LC^E$) (**Figure 19**).Then we compared the presentation kinetics, after adding the proteasome inhibitor LC, of the 6 epitopes derived from post-replicative viral genes with the kinetics of the model epitopes B8R and B2R. We established that three of the six epitopes resisted to different extents the LC treatment (A19L, A25L and A6L), and the other three were $LC^S$ (A3L, A42R and E7R) (**Figure 20**). Since protein synthesis strongly correlates with antigen presentation (Croft *et al.*, 2013), we can assume that the antigen presentation kinetics is a *bona fide* insight of protein synthesis, showing that there is some leakiness in post-replicative protein synthesis inhibition by LC treatment, as we detected some antigen presentation after treating infected cells with LC from the beginning of the infection (**Figure 20**).

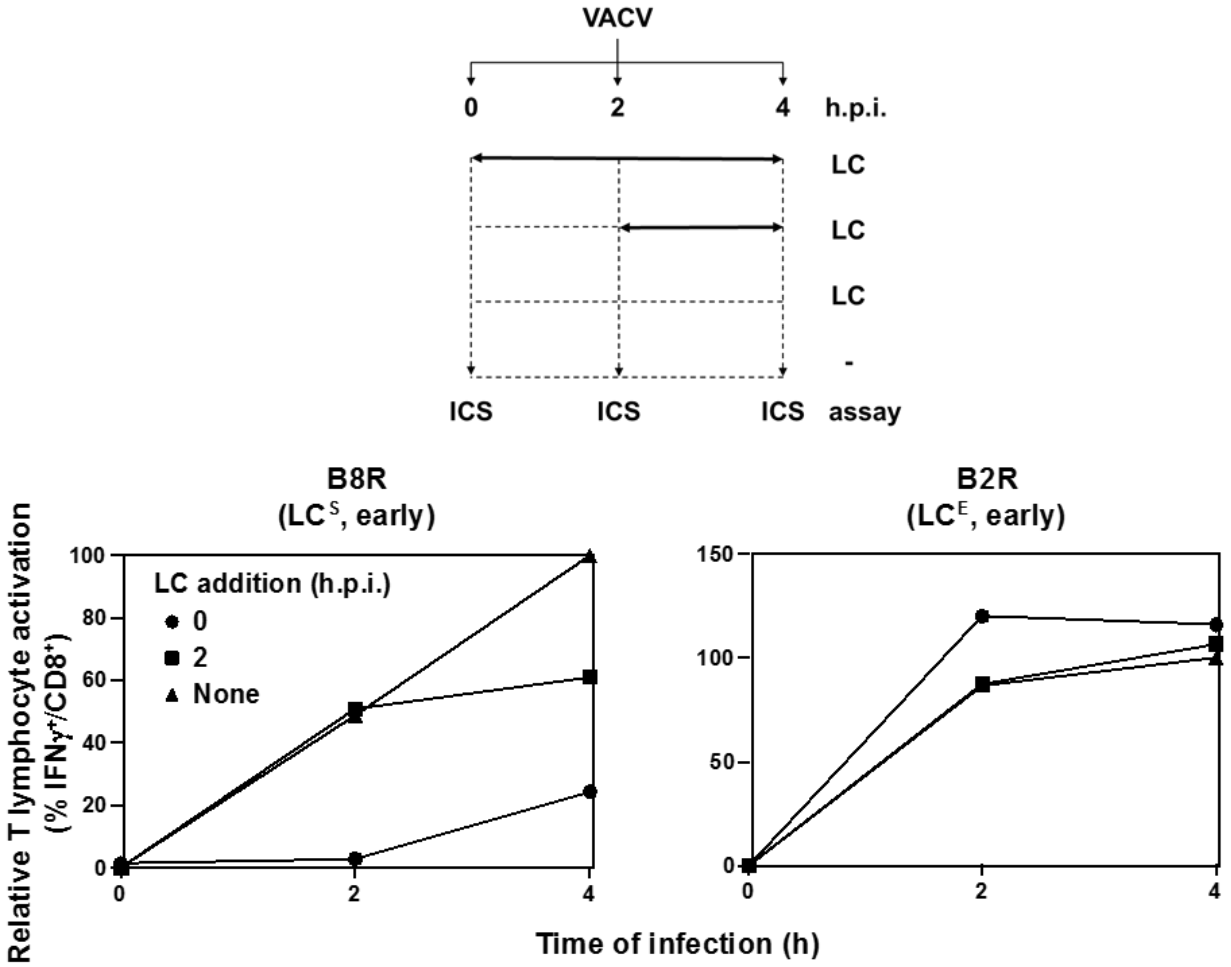



**Figure 19. Antigen presentation kinetics after adding LC reproduces known sensitivity to the inhibitor for the model epitopes B8R and B2R.**

C57BL/6 BMDC were infected with VACV WR for 0, 2 or 4 hours at 37ºC and the proteasome inhibitor LC was added at the beginning of the infection (●), 2 hours after the infection (■) or not at all (▲). These cells were used to stimulate CTL lines specific for the epitopes B8R and B2R and IFNγ production was measured by flow cytometry. Antigen presentation sensitivity to the inhibitor LC was assessed by comparing IFNγ production of CTL lines stimulated with BMDC infected for the same time but treated with LC for different hours. At all points, IFNγ production was relativized within the same experiment to IFNγ production of $CD8^+$ T lymphocytes stimulated with BMDC infected for the longest

time post-infection and not treated with LC.

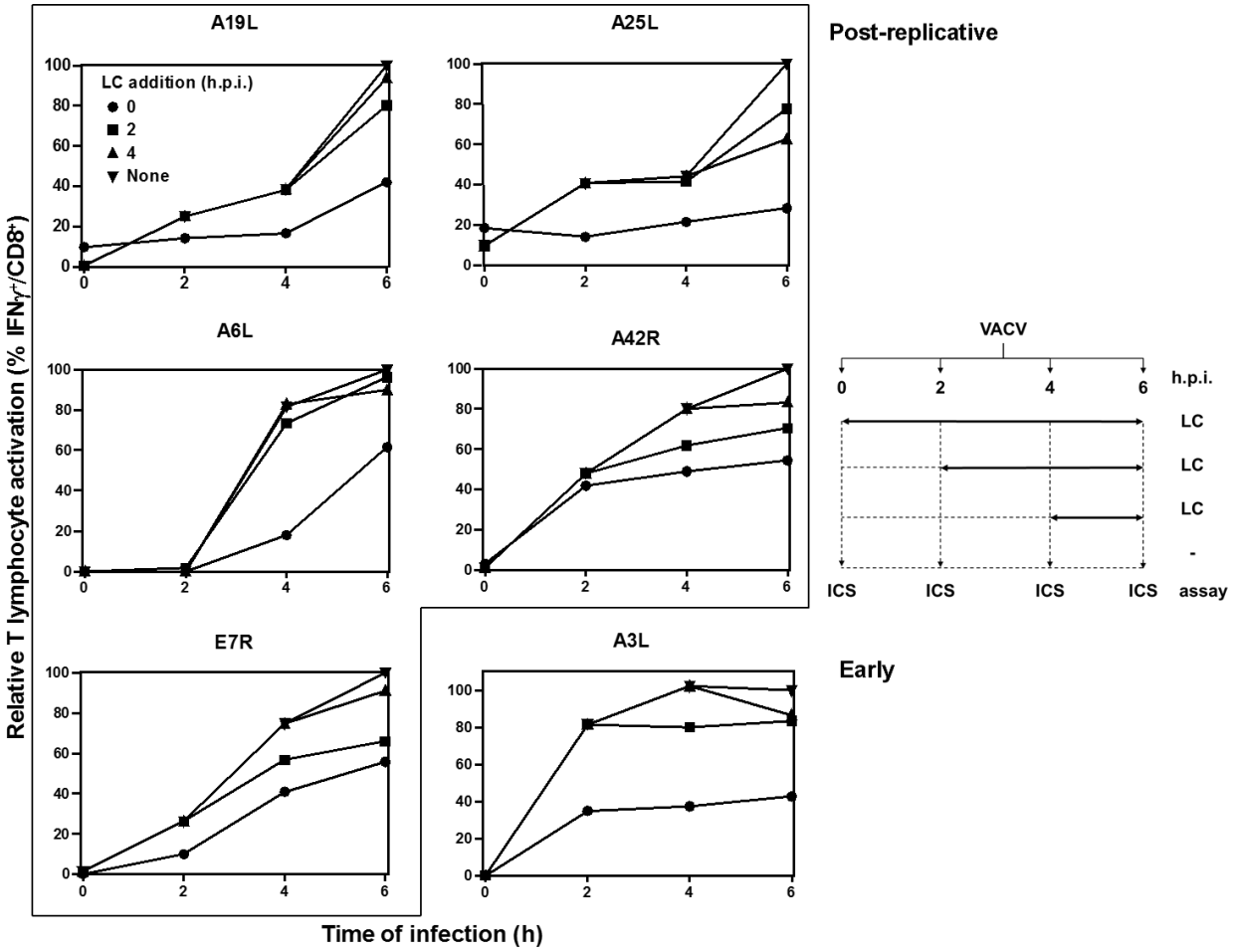



**Figure 20. Adding LC at different times post-infection allows assessing the requirement for proteasomes for the generation of epitopes derived from post-replicative expression genes.**

C57BL/6 BMDC were infected with VACV WR for 0, 2, 4 or 6 hours and were used to stimulate CTL lines specific for the TAP-independent and post-replicative epitopes A19L, A25L, A6L, A3L, A42R and E7R. The proteasome inhibitor LC was added at the beginning of the infection (●), 2 hours later (■), 4 hours later (▲) or not at all (▼). At all points, IFNγ production was relativized within the same experiment to IFNγ production of $CD8^+$ T lymphocytes stimulated with BMDC infected for the longest time post-infection and not treated with LC. One representative experiment out of 3 or 5 for each CTL line is shown.

Besides, as a side result, we could compare the antigen presentation kinetics of all six epitopes included in the experiment by taking together the data corresponding to non-treated cells (**Figure 21**). In this representation, we can observe that the presentation of A3L is earlier than the rest of the epitopes derived from late expression genes, which agrees with other evidence indicating that A3L is expressed in the early phase (personal communication).As antigen presentation more or less reflects source protein synthesis (Croft

*et al.*, 2013), we can interpret these results as an indicator of the temporal gene expression of these viral proteins. In fact, the epitope A42R showed a presentation kinetic similar to A3L, suggesting that the epitope A42R could be derived from an early gene as well (**Figure 21**).This proposed reclassification of the kinetic phase of expression for these two genes does not contradict the conclusions on sensitivity to LC, but simplifies their interpretation. In addition, these results are consistent with previous results obtained in our group on LC and A3L (Silvia Lázaro, personal communication).

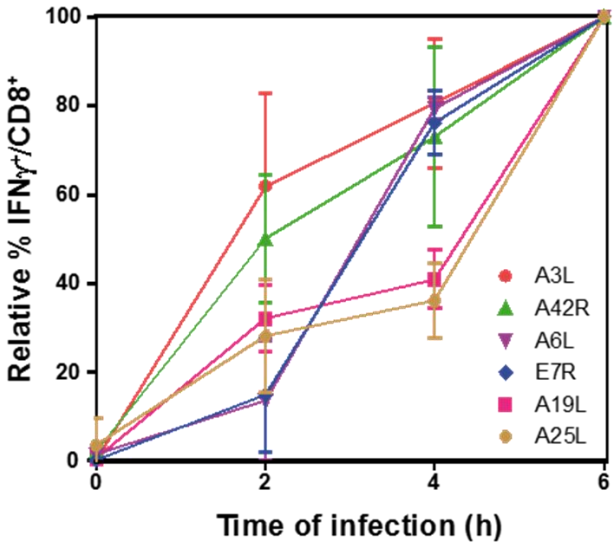



**Figure 21. TAP-independent epitopes derived from post-replicative expressed genes show great differences in the epitope presentation kinetics.**

C57BL/6 BMDC were infected for 0, 2, 4 or 6 hours with VACV WR and used to stimulate CTL lines specific for the TAP-independent epitopes A3L, A42R, A6L, E7R, A19L and A25L. IFNγ production was measured by flow cytometry and the relative $CD8^+$ T-lymphocyte activation was calculated for each epitope separately according to the formula: (activation at "t" h.p.i. /activation at 6 h.p.i.) x100. The data show the mean and SEM of two or three experiments for each CTL line.

| Epitope | TAP requirement | Temporal expression | Proteasome inhibitors sensitivity | |
|---|---|---|---|---|
| | | | C57BL/6 BMDCxVACV | $TAP1^{-/-}$ BMDCxVACV |
| **A19L (47-55)** | ind | Post-replicative | **$LC^R$** | - |
| **A23R (297-305)** | ind | Early | $LC^S$ | - |
| **A25L (257-264)** | ind | Post-replicative | **$LC^R$** | - |
| **A38L (203-210)** | ind | Post-replicative | - | - |
| **A3L (270-277)** | dep | Post-replicative | **$LC^S$** | - |
| **A42R (88-96)** | dep | Post-replicative | **$LC^S$** | - |
| **A47L (138-146)** | dep | Early | $LC^S$ | - |
| **A51R (78-85)** | dep | Early | $LC^R$ | - |

| | | | | |
|---|---|---|---|---|
| **A6L (265-272)** | ind | Post-replicative | **$LC^R$** | - |
| **A8R (189-196)** | ind | Early | $LC^S$ | $LC^S$ |
| **A8R (70-77)** | dep | Early | $LC^S$ | - |
| **B16R (275- 283)** | dep | Post-replicative | - | - |
| **B1R (92-99)** | dep | Early | $LC^S$ | - |
| **B2R (54-62)** | dep | Early | $LC^E$ | - |
| **B6R (108-116)** | dep | Early | $LC^S$ | - |
| **B8R (20-27)** | dep | Early | $LC^S$ | - |
| **C4L (125-132)** | dep | Early | $LC^S$ | - |
| **D1R (282-290)** | dep | Early | - | - |
| **D1R (578-586)** | dep | Early | $LC^R$ | - |
| **E7R (130-137)** | dep | Post-replicative | **$LC^S$** | - |
| **E8R (141-150)** | ind | Post-replicative | - | - |
| **F13L (307-315)** | ind | Post-replicative | - | - |
| **F1L (200-207)** | dep | Early | $LC^S$ | - |
| **F5L (279-287)** | ind | Early | - | $Epo^S$ |
| **G8R (34-41)** | ind | Post-replicative | - | - |
| **J3R (289-296)** | ind | Early | - | $LC^S$ |
| **J6R (993-1000)** | dep | Early | $LC^S$ | - |
| **K3L (6-15)** | dep | Early | $LC^S$ | - |
| **L2R (53-61)** | ind | Early | $LC^S$ | $LC^S$ |
| **L2R (61-69)** | dep | Early | - | - |

**Table 5. VACV MHC-I restricted epitopes included in this study.**

The 30 MHC-I restricted epitopes recognized by $CD8^+$ T lymphocytes that were previously studied in our lab (Silvia Lázaro et al., in preparation) and their characteristics regarding their need for TAP(dep, dependent; ind, independent), the temporal expression of the genes that encode them and the sensitivity of their presentation after the addition of LC ($LC^S$, sensitive; $LC^R$, resistant; $LC^E$, enhanced) and Epoxomicin (Epo) are summarized. Dashes (-) indicate not tested. Results obtained in this work are marked in bold.

To compare the role of proteasomes in both global (TAP-dependent and TAP-independent together) and TAP-independent antigen presentation alone, we compiled in **Table 5** all the data in the lab (Silvia Lázaro et al., in preparation) and data from **Figure 20** and classified the epitopes whose presentation was sensitive ($LC^S$), resistant ($LC^R$) or enhanced by the treatment with LC ($LC^E$) (**Figure 22**). From the 18 TAP-dep epitopes, while 12 epitopes showed sensitivity to the treatment with LC, 2 epitopes resisted the treatment and the presentation of one epitope was enhanced by this inhibitor. These 3 epitopes that do

not show sensitivity to LC may account for at least part of the TAP-dependent viral antigen processing resistance to LC in **Figure 16A.** On the other hand, we detected TAP-ind viral antigen presentation resistant to LC in **Figure 16B** while we failed in finding individual epitopes resistant to LC in TAP1$^{-/-}$ BMDC. This can be due to an incomplete inhibition of proteasomal activity in **Figure 16B** or to the limited number of epitopes tested until now in TAP1$^{-/-}$ BMDC (4 out of 12). Three potential TAP-ind and LC$^{R}$ epitopes are A19L, A25L and A6L, which showed resistance to LC in C57BL/6 BMDC and are presented in TAP1$^{-/-}$ BMDC (**Table 5**). However, most of the epitopes tested showed sensitivity to LC, which strongly agrees with the results obtained in ex vivo experiments (**Figure 16A and B**).

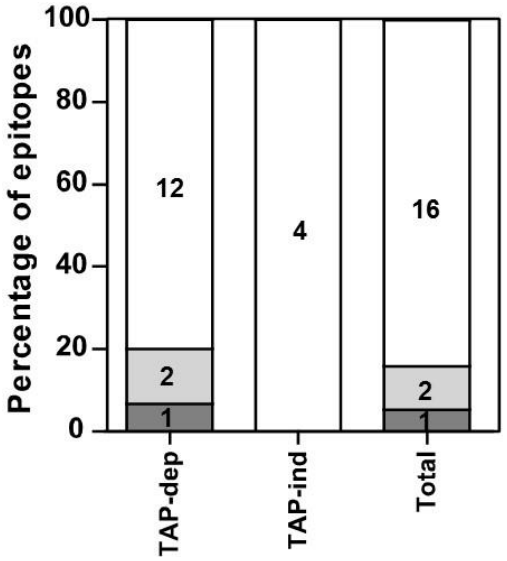



**Figure 22. Role of proteasomes in the processing of TAP-dep and TAP-ind VACV antigens.**

The number of epitopes according to their requirement for proteasomal processing is shown (numbers in bars). The percentage of epitopes within each group (TAP-dep, TAP-ind and Total) is represented on the vertical axis.

### 5.2.3. Role of non-proteasomal proteolytic enzymes in the processing of VACV antigens.

Knowing that proteasomes play the major role in TAP-independent antigen presentation and that there are also epitopes that are generated in the presence of a proteasome inhibitor in TAP-proficient cells, we aimed to know what protease or proteases participate in this kind of alternative antigen presentation pathways.

We chose a battery of protease inhibitors with different specificities (**Table 6**). None of these inhibitors showed a negative effect on the expression of GFP in BMDC infected with a VACV expressing GFP (**Figure 23**).

| Inhibitor | Abbreviation | Specificity | Concentration of use | Reference |
|---|---|---|---|---|
| **Lactacystin** | LC | Proteasomes | 10 µM | (Fenteany *et al.*, 1995) |
| **Captopril** | - | Angiotensin-converting enzyme | 150 µM | (Looker *et al.*, 2015b) |
| **Decanoyl-Arg-Val-Lys-Arg-chloromethylketone** | dec-RVKR-cmk | Proprotein convertases | 50 µM | (Garten *et al.*, 1994) |
| **Leucinthiol** | LeuSH | Metalloaminopeptidases | 30 µM | (Schumacher and Schreiber, 2015) |
| **N-Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone** | z-VAD-fmk | Caspases | 100 µM | (Zhu *et al.*, 1995) |

**Table 6. Characteristics of the inhibitors of proteolytic activity used in this study.**

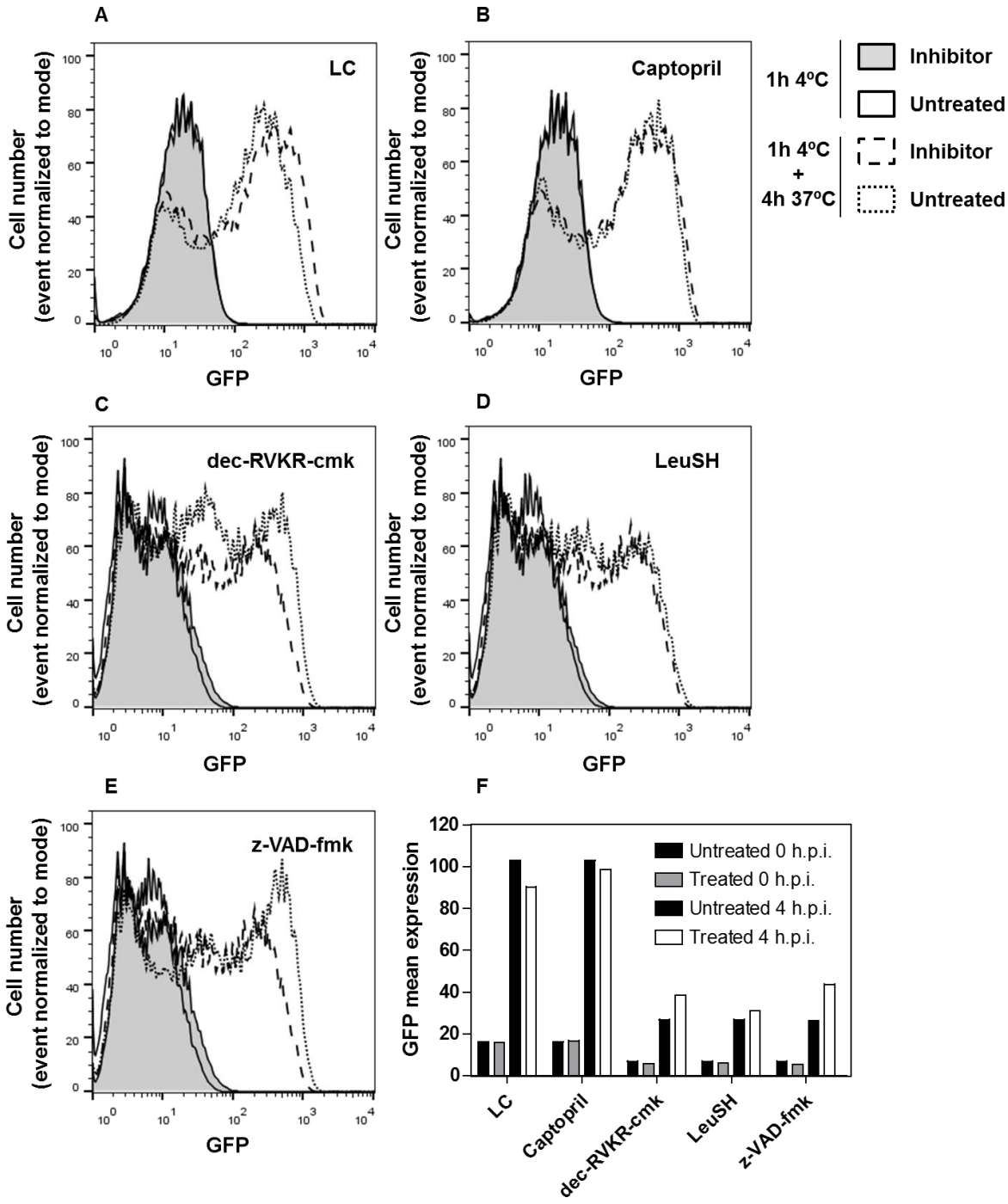



**Figure 23. The protease inhibitors lactacystin, captopril, dec-RVKR-cmk, leucinthiol and z-VAD-fmk do not impair VACV gene expression after infection of BMDC.**

BMDC were infected with rVV-GFP and treated or not with lactacystin (**A**), captopril (**B**), dec-RVKR-cmk (**C**), leucinthiol (**D**) or z-VAD-fmk (**E**) during the infection. To assess the possible toxicity of the inhibitor during a VACV infection, GFP expression was measured by flow cytometry after one hour adsorption at 4ºC with (filled histogram) or without (continuous line, empty histogram) inhibitor (0 h.p.i.) and after one hour at 4ºC and four more hours at 37ºC with (dashed line) or without (dotted line) inhibitor (4 h.p.i.). In all cases, GFP mean fluorescence intensity was calculated and graphed (**F**).

We then established specific CTL lines for TAP-independent epitopes and performed ICS assays stimulating them with VACV-infected BMDC treated or not with each protease inhibitor. Then, we calculated the percentage of inhibition of antigen presentation. The results showed that only the proprotein convertases inhibitor dec-RVKR-cmk and the metalloaminopeptidases inhibitor LeuSH had an effect on the presentation of some epitopes included in the experiments (**Figure 24A**).

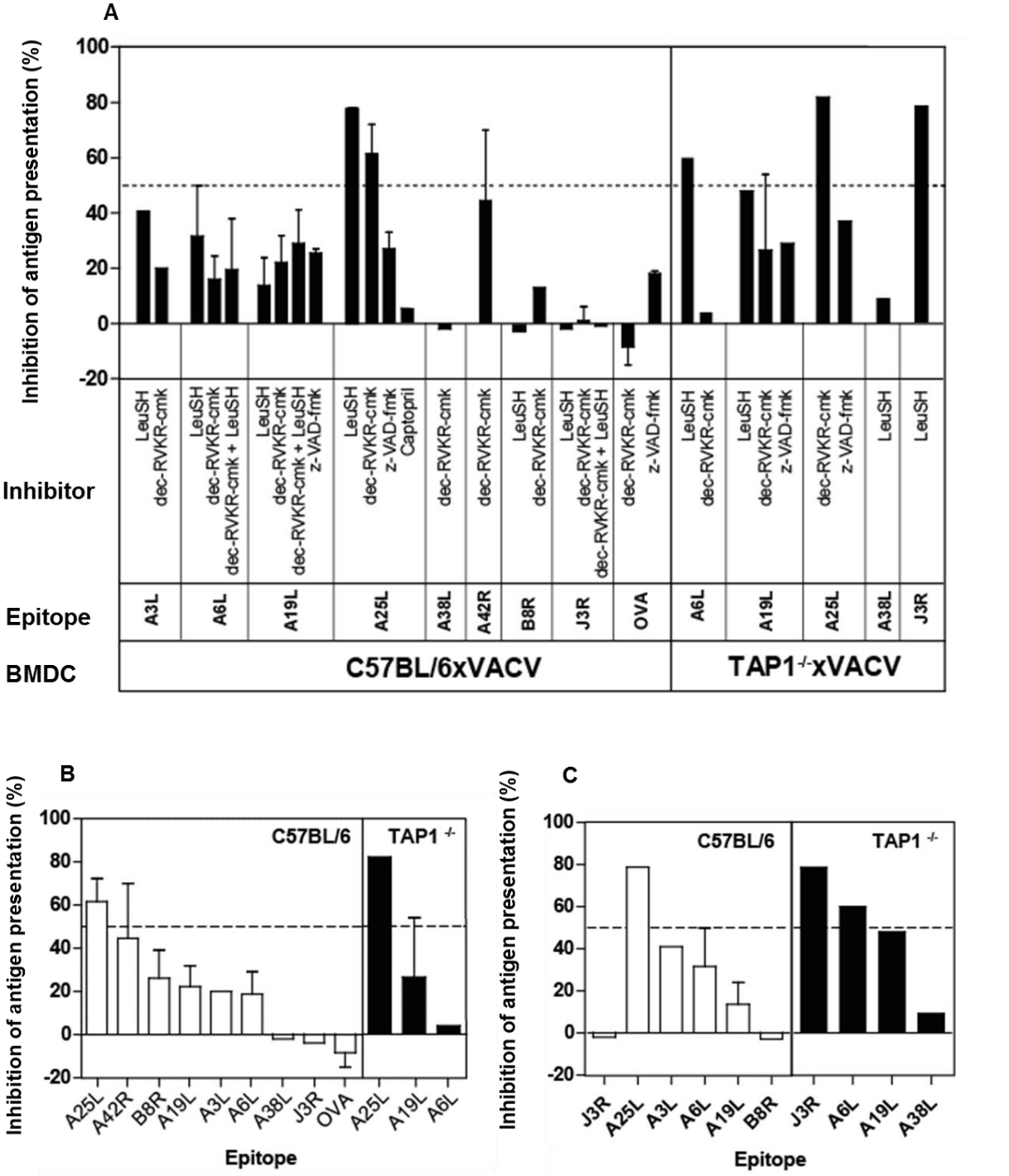



**Figure 24. Inhibition of antigen presentation by protease inhibitors LeuSH, dec-RVKR-cmk, z-VAD-fmk and Captopril.**

C57BL/6 and TAP1$^{-/-}$ BMDC were infected by a recombinant VACV encoding cytosolic OVA and treated or not with the protease inhibitors LeuSH, dec-RVKR-cmk, z-VAD-fmk and Captopril together or separately as indicated (**A**). After 6 hours of infection, BMDC were cocultured with epitope-specific CTL specific lines and CD8$^{+}$ T-lymphocyte activation was measured by flow cytometry as an indicator of antigen presentation by MHC-I by infected cells. We included OVA as well as other VACV epitopes. Besides, we checked that the treatment with every inhibitor did not reduce the presentation of limiting amounts of each synthetic peptides (data not shown). For a better comprehension, data corresponding only to the treatment with dec-RVKR-cmk (B) and LeuSH (C) are graphed separately (**B**). Percentage inhibition of antigen presentation was calculated for each epitope and treatment, following the formula: (1-(activation against treated cells/activation against non-treated cells)) x100. Mean and SEM of 1 to 7 independent experiments are shown. The dashed line marks 50% inhibition as an arbitrary indicator of relevant inhibition.

For better comprehension, we graphed the results corresponding only to the BMDC treated with dec-RVKR-cmk (**Figure 24B**) or LeuSH (**Figure 24C**). We considered a relevant inhibition only if it exceeded 50% and with that criterion, the only epitope affected by dec-RVKR-cmk treatment in VACV-infected C57BL/6 BMDC was the TAP-independent epitope A25L. Although with only one experiment, when the CTL are stimulated with infected TAP1$^{-/-}$ BMDC the only epitope inhibited by dec-RVKR-cmk is also A25L,, suggesting that proprotein convertases might participate in the TAP-independent antigen presentation pathway of this epitope. The fact that we are able to detect inhibition in C57BL/6 infected BMDC suggests either (1) that proprotein convertases not only might participate in the TAP-independent route, but also in the TAP-dependent pathway of this epitope, or (2), if they would only participate in the TAP-independent route, this route may have a major participation in the generation of this epitope, even in the presence of TAP. Results corresponding to non-treated BMDC in **Figure 24**using CTL lines specific for A25L (not shown) indicated that, for this particular epitope, TAP-independent pathways would contribute around 19% to the total presentation of the epitope. These data would support the first hypothesis to explain the antigen presentation inhibition by dec-RVKR-cmk in infected C57BL/6 BMDC.

Also, the inhibition shown after treatment with LeuSH suggests that aminopeptidases, presumably ERAP1 and/or ERAP2, might have a relevant role in antigen processing in the absence of TAP, since 2 out of 4 epitopes tested exceed the threshold of 50 % inhibition (**Figure 24C**). If that were the case, TAP-ind antigens would access the ER somehow during their processing.

## 5.3. Physiological relevance of TAP-independent antigen presentation pathways.

### 5.3.1. Viral clearance kinetics in TAP1$^{-/-}$ mice.

Once we described the role of proteasomes in TAP-independent antigen presentation and observed resistance to LC in TAP-proficient infected cells for some epitopes, we wondered if this kind of alternative pathways were relevant for viral clearance *in vivo*. With that purpose, we first studied the viral clearance kinetics in C57BL/6 and TAP1$^{-/-}$ mice. In particular, we measured the viral load in ovaries (**Figure 25A**) and spleens (**Figure 25B**) at different days after a sublethal VACV infection. The results show that initial viral replication was indistinguishable between TAP1$^{-/-}$ and C57BL/6 mice. Also, TAP1$^{-/-}$ mice cleared the infection in both organs as efficiently as C57BL/6 mice (**Figure 25**).

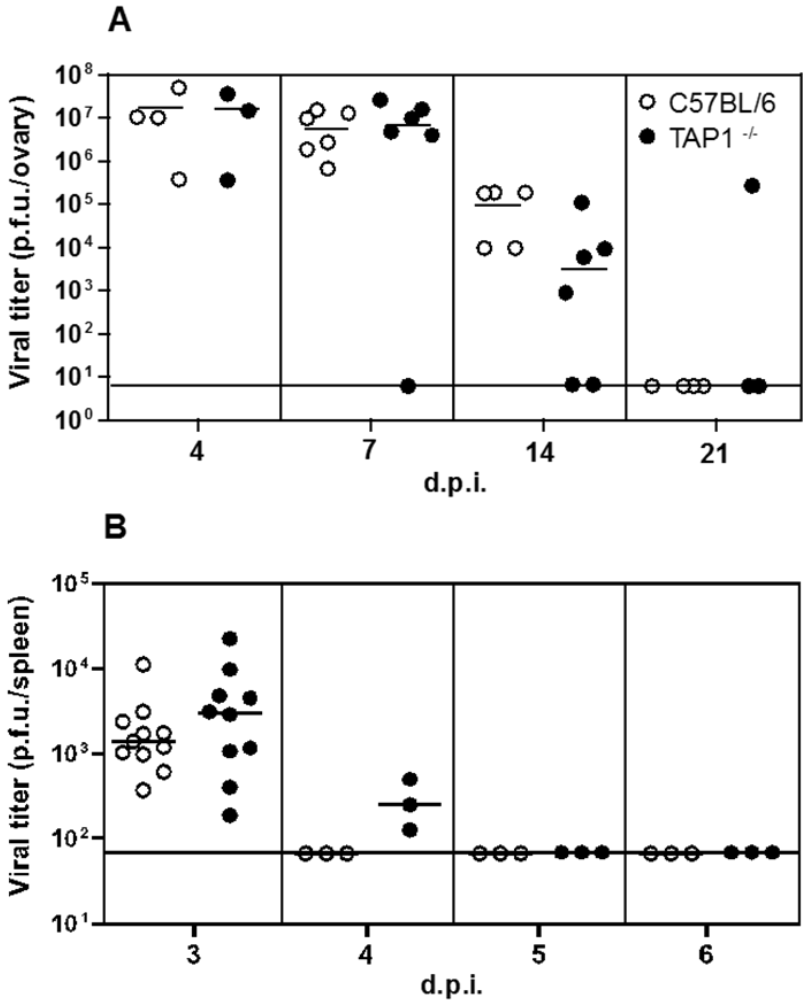



**Figure 25. TAP1$^{-/-}$ mice clear VACV *in vivo* as efficiently as C57BL/6 mice.**

C57BL/6 and TAP1$^{-/-}$ mice were infected with 5·10$^6$PFU of VACV WR and viral titers were measured at different days post-infection. Each point represents either the mean titer of two ovaries of the same

mouse (**A**) or the viral titer of the whole spleen (**B**). Three to eleven mice per group were used. Short lines indicate the median viral load of each group. The long black line indicates the detection limit of the viral titration assay.

### 5.3.2. Involvement of $CD8^+$ T lymphocytes in TAP-independent viral clearance.

Knowing that VACV infection follows the same kinetics in both C57BL/6 and $TAP1^{-/-}$ mice, we wanted to know whether TAP-independent antigen presentation routes participate in the elimination of VACV-infected cells mediated by $CD8^+$ T lymphocytes. With that purpose, we chose three TAP-ind epitopes to perform TAP-ind vaccination experiments (**Table 7**).

| Epitope | TAP requirement | Requirement for proteasomal activity | | Other proteases involved |
|---|---|---|---|---|
| | | C57BL/6 BMDCxVACV | $TAP1^{-/-}$ BMDCxVACV | |
| **J3R** | Ind* | - | Yes* | n.d. |
| **A19L** | Ind* | No | - | n.d. |
| **A25L** | Ind* | No | - | Proprotein convertases |
| **B8R** | Dep* | Yes* | - | n.d. |

**Table 7. Characteristics of the epitopes regarding alternative antigen processing pathways included in *in vivo* experiments.**

The four epitopes included in further experiments are summarized in this table as well as the requirement for TAP, proteasomes and other known proteases. (*) Silvia Lázaro et al., in preparation.

We inoculated C57BL/6 and $TAP1^{-/-}$ mice intraperitoneally with $TAP1^{-/-}$ BMDC loaded with one of the synthetic peptides B8R, J3R, A19L or A25L. We included the epitopes J3R and B8R in the experiments due to their immunodominant role in the anti-VACV $CD8^+$ lymphocyte response of $TAP1^{-/-}$ (J3R) and C57BL/6 mice (B8R). Fourteen days later, we infected the mice with $5 \cdot 10^6$ PFU of VACV and the viral clearance after the infection was tracked until the viral load in the ovaries was under the limit of detection. As we wanted to achieve a substantial viral load, we infected with a moderately high dose of virus, since it is known that higher doses lead to higher viral loads (Lin *et al.*, 2013).The results show some improvement of viral clearance by $CD8^+$ T lymphocytes in C57BL/6 mice vaccinated with the peptides J3R or A19L, as well as the immunodominant epitope B8R. However, $TAP1^{-/-}$mice are not protected by these peptides (**Figure 26**).

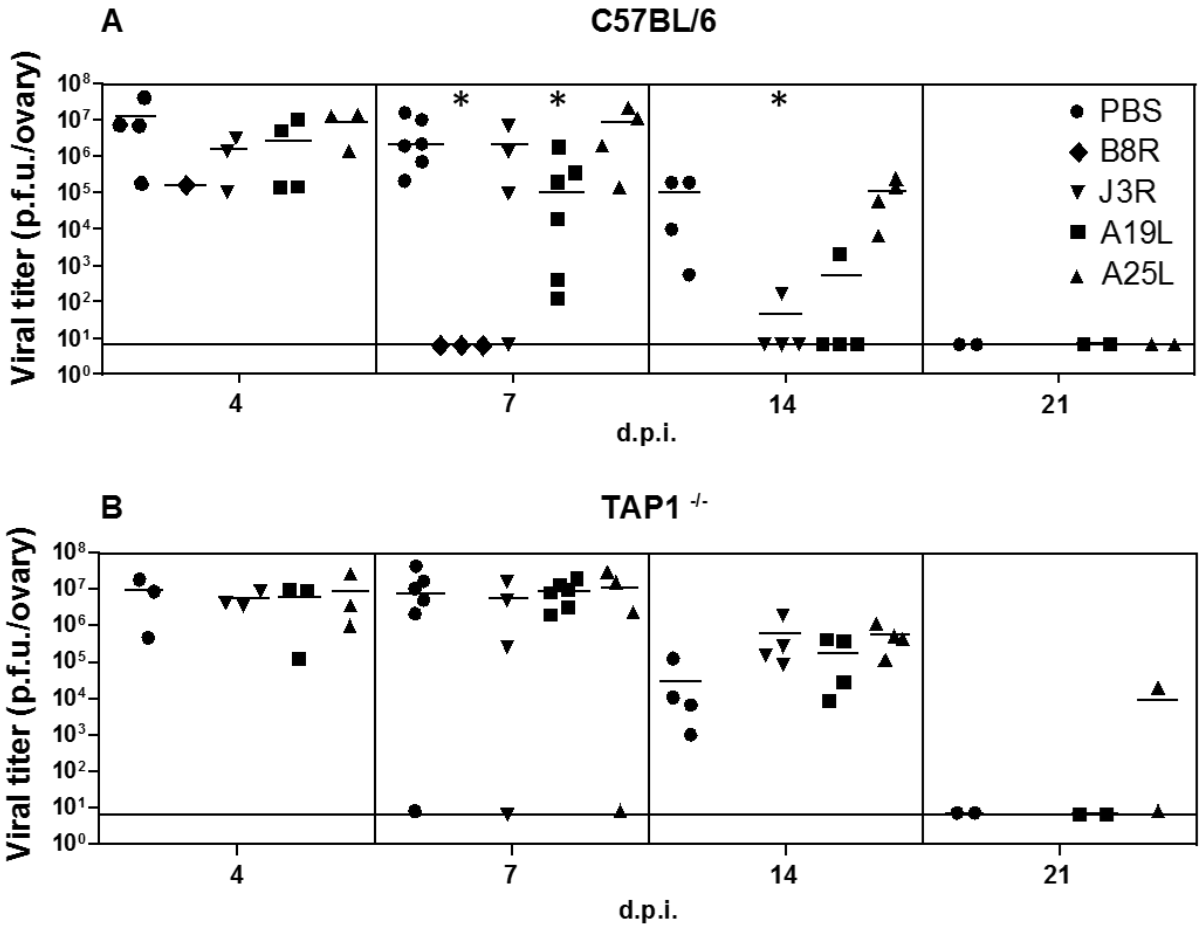



**Figure 26. Vaccination with J3R and A19L but not with A25L improves virus clearance from ovaries in C57BL/6 mice but not in TAP1$^{-/-}$ mice.**

C57BL/6 (**A**) and TAP1$^{-/-}$ (**B**) mice were inoculated i.p. with LPS-matured BMDC previously incubated with the indicated synthetic peptides or with PBS alone as a negative control. Fourteen days later, mice were infected i.p. with $5 \cdot 10^6$PFU of VACV WR. Viral load in the ovaries was measured 4, 7, 14 and 21 days after the infection. The mean load of two ovaries of each mouse was calculated and graphed as an individual spot for each mouse. Short lines indicate the median viral load of each group. The long black line indicates the detection limit of the viral titration assay. Mann-Whitney U tests to compare difference of medians of viral load taking as a control PBS-incubated mBMDC were performed for each day post-infection (p value ≤ 0.001, ***; p value ≤ 0.005, **; p value ≤ 0.05, *).

We performed the same experiment tracking the viral load in the spleens. These results showed an improved capacity of CD8$^+$ T lymphocytes to clear the infection after vaccination with J3R, A19L or A25L synthetic peptides in TAP1$^{-/-}$ mice, while only the vaccination with the epitopes J3R and A19L showed significant protection in C57BL/6 mice (**Figure 27**). The fact that the inoculation consisted in synthetic peptides that are recognized by CD8$^+$ T lymphocytes proves that this improved clearance after vaccination is due to the sole action of these cells.

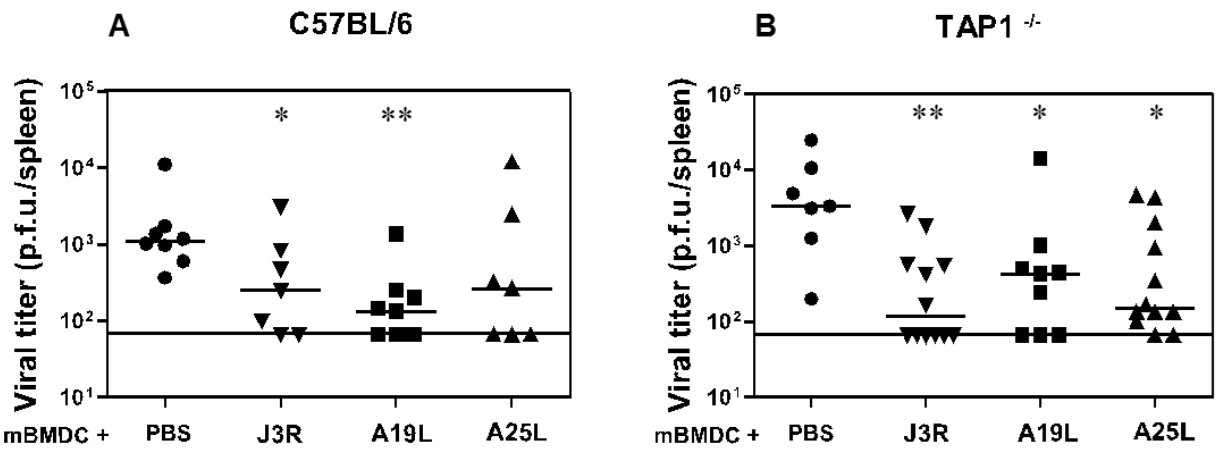



**Figure 27. The TAP-independent epitopes J3R and A19L confer protection against a VACV infection *in vivo* in both C57BL/6 and TAP1$^{-/-}$ mice.**

C57BL/6 (**A**) and TAP1$^{-/-}$ (**B**) mice were inoculated with LPS-matured BMDC loaded with the indicated synthetic antigenic peptides. Fourteen days later, mice were infected with $5{\cdot}10^6$PFU of VACV WR and three days after infection, spleens were extracted to measure the viral titer. The black line indicates the detection limit of the viral titer assay. Data from four independent experiments with a total of at least seven mice per group is shown. Mann-Whitney U tests to compare difference of medians of viral load taking as a control PBS-incubated mBMDC were performed (p value ≤ 0.001, ***; p value ≤ 0.01, **; p value ≤ 0.05, *).

However, we also performed ICS assays after challenge infection to check the cell response itself. With the exception of A19L and A25L in TAP1$^{-/-}$ mice, all the inoculations led to an increased CD8$^+$ response against the inoculated peptide, compared to mice that were inoculated only with mature BMDC (mBMDC). We also detected a higher background response to most peptides in TAP1$^{-/-}$ mice (**Figure 28**). We detected a lesser percentage of CD8$^+$ T lymphocytes in TAP1$^{-/-}$ mice compared to C57BL/6 mice in both PEC and spleens (3.8 % ± 2.2 % CD8$^+$ lymphocytes in TAP1$^{-/-}$*versus* 14.2 % ± 5.1 % in C57BL/6 PEC and 1.8 % ± 0.7 % in TAP1$^{-/-}$*versus*17.1 % ± 5.3 % in C57BL/6 spleens).This agrees with previously described similar data in uninfected mice (van Kaer *et al.*, 1992).

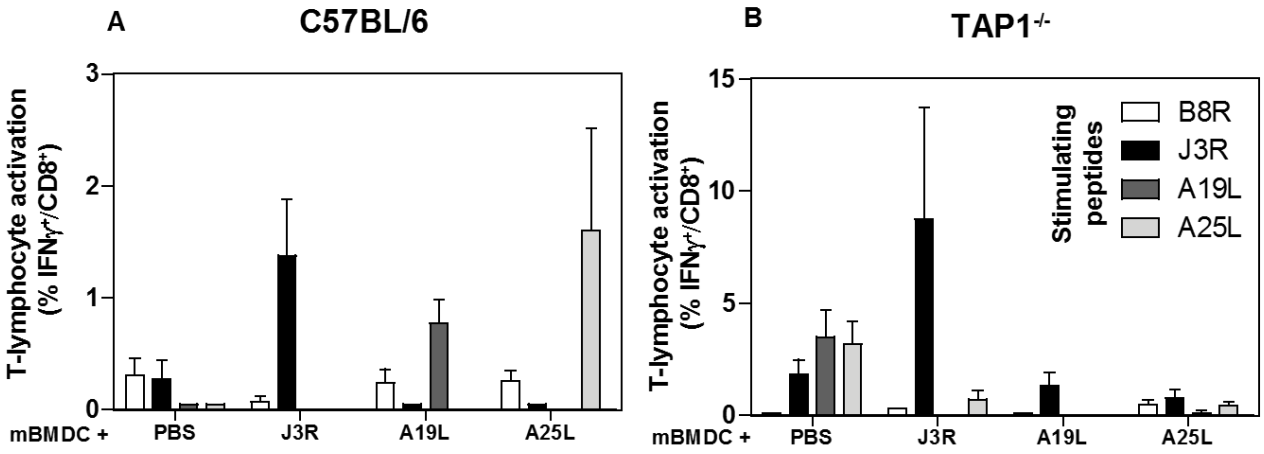



**Figure 28. Immunization with mBMDC loaded with antigenic peptides leads to an enhanced CD8$^+$ T lymphocyte response after viral infection.**

C57BL/6 (**A**) or TAP1$^{-/-}$ (**B**) mice were immunized with mBMDC loaded either with PBS or with J3R, A25L or A19L antigenic peptides. Fourteen days later mice were challenged with 5·10$^6$PFU of VACV and three days after infection the CD8$^+$ T lymphocyte response from PEC against each peptide (B8R, white; J3R, black; A19L, dark grey; A25L, light grey) was measured in an ICS assay.

The effective viral clearance by CD8$^+$ T lymphocytes after vaccination in spleens but not in ovaries in the absence of TAP shows that alternative antigen processing pathways have differential physiological relevance to clear a VACV infection depending on other factors in addition to the presence of TAP. To clarify this question, we hypothesized that MHC-I density may underlie the differences in protection between ovaries and spleens. H-2K$^b$ binds and presents to CD8$^+$ T lymphocytes the peptides summarized in **Table 7**. Therefore, we measured by flow cytometry the H-2K$^b$surface expression in C57BL/6 and TAP1$^{-/-}$ ovaries and spleens (**Figure 29**). The results showed a negative correlation between MHC-I expression and viral load in C57BL/6 mice, being the spleen the organ with more MHC-I and less viral load at early days post-infection. This is consistent with higher MHC-I levels contributing to better CD8$^+$ T lymphocyte-mediated control of virus. However, this cannot be concluded about TAP1$^{-/-}$ mice, since both ovaries and spleens show a similar MHC-I expression, despite very different viral titers (**Figure 29B**).The lower MHC-I surface expression in TAP1$^{-/-}$ mice was already described(van Kaer *et al.*, 1992). Therefore, MHC-I expression alone does not explain the differences observed in the results regarding protection against VACV.

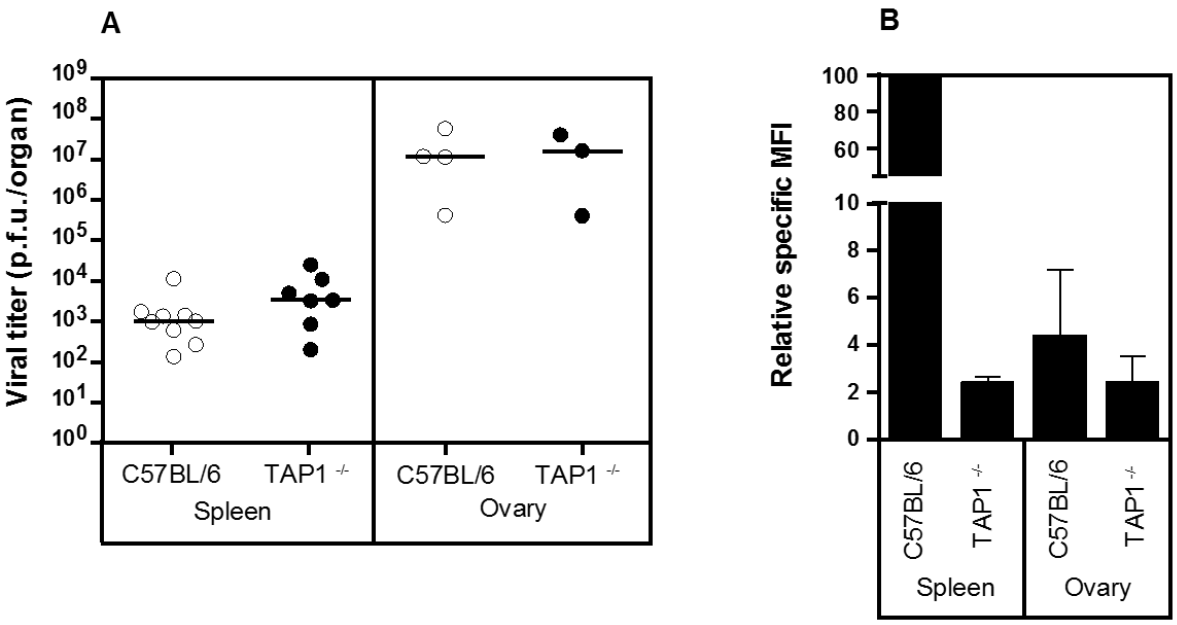



**Figure 29. At early days post-infection ovaries have more virus than spleens which correlates with a lower MHC-I surface expression in C57BL/6 mice, but not in TAP1$^{-/-}$ mice.**

C57BL/6 and TAP1$^{-/-}$ mice were infected with VACV WR and viral load was measured at day 3 (for spleens) or day 4 (for ovaries) post-infection. Dots represent individual mice, corresponding to the same data from previous experiments (**A**). Spleens and ovaries were extracted from uninfected C57BL/6 and TAP1$^{-/-}$ mice and surface H-2K$^b$ expression was measured. Fluorescence from cells incubated with isotype control was subtracted to obtain the specific MFI and data was relativized within the same experiment to cells with the highest MFI signal (C57BL/6 splenocytes). Data from 3 independent experiments is shown (**B**). Medians of each group are shown by black lines in A, while means and SEM are shown in B.

## 5.4. Differential features of VACV TAP-independent epitopes.

### 5.4.1. Hydrophobicity of VACV immunogenic epitopes as a potential driver of TAP independence.

After studying the antigen presentation mechanisms and the relevance of TAP-independent routes, we wondered which features conferred a peptide the capability of being processed and presented in a TAP-independent manner. A previous work that studied the TAP requirement of nine epitopes derived from the Epstein Barr virus (EBV) LMP2 protein identified a correlation between the hydrophobicity of the epitopes and their requirement for TAP to be presented by HLA molecules, being the TAP-independent epitopes the most hydrophobic (Lautscham *et al.*, 2003) (**Table 8**). We calculated the hydrophobicity score using the optimal matching hydrophobicities calculation (OHM) which is a value used to

predict conformational similarities of proteins from their aa sequences. Numerical values for hydrophobicity, OHMs, are assigned to each aa residue to allow comparisons based upon features of the residues that are important for their structural function, but independent of actual aa identities(Sweet and Eisenberg, 1983).

| Epitope | HLA restriction | Hydrophobicity (OHM score) | Relative hydrophobicity* | TAP requirement | Epitope location |
|---|---|---|---|---|---|
| **LLWTLVVLL** | A*0201 | 8,14 | 0,90 | ind | TM |
| **FLYALALLL** | A*0201 | 7,64 | 0,84 | ind | TM |
| **WTLVVLLI** | B*63 | 6,95 | 0,87 | ind | TM |
| **PYLFWLAAI** | A*23 | 6,49 | 0,72 | ind | TM |
| **TYGPVFMCL** | A*2402 | 5,47 | 0,61 | ind | 4 aa Cytosol + 5 aa TM |
| **CLGGLLTMV** | A*0201 | 4,14 | 0,46 | ind | TM |
| **IEDPPFNSL** | B*4001 | -0,59 | -0,07 | dep | Lumen |
| **RRRWRRLTV** | B*2704 | -0,60 | -0,07 | dep | Cytosol |
| **SSCSSCPLSK** | A*1101 | -2,35 | -0,24 | dep | Cytosol |

**Table 8. Epitopes from the EBV protein LMP2 included in Lautscham et al., 2003.**
The epitopes studied in Lautscham et al, 2003 are summarized and their hydrophobicity and TAP status are shown (dep, dependent; ind, independent).TAP-ind epitopes are colored in grey. *Relative hydrophobicity is calculated by dividing the OHM hydrophobicity score by the number of aa in the peptide sequence. TM: transmembrane, aa: aminoacid.

Besides, the six TAP-independent epitopes were part of transmembrane (TM) regions. The fact that the eight epitopes derive from the same protein, suggest that the requirement for TAP is driven by a feature of the peptide or a precursor rather than a feature of the protein (LMP2). Also, the observation that the TAP-independent epitopes are part of TM regions suggests a question: Is the high hydrophobicity a cause of their TAP-independency or is it only a consequence of their TM location?

To answer that question, we would need a high variety of TAP-independent epitopes derived from different proteins and secondary structures. Previous results in our lab provide that tool, since 30 MHC-I restricted epitopes encoded by VACV were tested for their requirement of TAP (Silvia Lázaro, personal communication). Twelve out of thirty were presented in a TAP-independent manner and only five of them contained at least one aa in a TM region ( **Table *9***).

| Epitope | Sequence | H-2 restriction | Hydrophobicity (OHM score) | Relative hydrophobicity* | TAP requirement | Epitope location |
|---|---|---|---|---|---|---|
| **L2R (53-61)** | VIYIFTVRL | **$K^b$** | 8,26 | 0,92 | ind | 9 aa TM |
| **A8R (189-196)** | ITYRFYLI | **$K^b$** | 8,11 | 1,01 | ind | Cyt |
| **G8R (34-41)** | LMYIFAAL | **$K^b$** | 7,50 | 0,94 | ind | 5 TM aa + 3 Cyt aa |
| **A23R (297-305)** | IGMFNLTFI | **$D^b$** | 6,71 | 0,75 | ind | Cyt |
| **A38L (203-210)** | KVFSFWLL | **$K^b$** | 6,47 | 0,81 | ind | 6 TM aa + 2 Cyt aa |
| **D1R (282-290)** | LGYIIRYPV | **$D^b$** | 6,22 | 0,69 | dep | Cyt |
| **A8R (70-77)** | IHYLFRCV | **$K^b$** | 5,91 | 0,74 | dep | Cyt |
| **B6R (108-116)** | LMYDIINSV | **$K^b$** | 5,54 | 0,62 | dep | Cyt |
| **J3R (289-296)** | SIFRFLNI | **$K^b$** | 5,50 | 0,69 | ind | Cyt |
| **J6R (993-1000)** | INFEFVCL | **$K^b$** | 5,25 | 0,66 | dep | Cyt |
| **A6L (265-272)** | YTLIYRQL | **$K^b$** | 5,25 | 0,66 | ind | Cyt |
| **A25L (257-264)** | SIYQYVRL | **$K^b$** | 4,67 | 0,58 | ind | Luminal |
| **A3L (270-277)** | KSYNYMLL | **$K^b$** | 4,66 | 0,58 | dep | Cyt |
| **E8R (141-150)** | FWFKNTQFDI | **$D^b$** | 4,42 | 0,44 | ind | 3 TM aa + 7 Cyt aa |
| **B1R (92-99)** | INVEYRFL | **$K^b$** | 4,24 | 0,53 | dep | Cyt |
| **A42R (88-96)** | YAPVSPIVI | **$D^b$** | 4,06 | 0,45 | dep | Cyt |
| **A19L (47-55)** | VSLDYINTM | **$K^b$** | 4,01 | 0,45 | ind | Cyt |
| **D1R (578-586)** | SMYCSKTFL | **$D^b$** | 3,95 | 0,44 | dep | Cyt |

| | | | | | | |
|---|---|---|---|---|---|---|
| **B16R (275-283)** | ISVANKIYM | **$D^b$** | 3,56 | 0,40 | dep | Luminal |
| **L2R (61-69)** | LVSRNYQML | **$K^b$** | 3,07 | 0,34 | dep | 2 TM aa |
| **A47L (138-146)** | AAFEFINSL | **$K^b$** | 2,82 | 0,31 | dep | Cyt |
| **C4L (125-132)** | LNFRFENV | **$K^b$** | 2,32 | 0,29 | dep | Cyt |
| **B2R (54-62)** | YSQVNKRYI | **$D^b$** | 1,86 | 0,21 | dep | Cyt |
| **K3L (6-15)** | YSLPNAGDVI | **$D^b$** | 1,71 | 0,17 | dep | Cyt |
| **A51R (78-85)** | RISRFANL | **$K^b$** | 1,34 | 0,17 | dep | Cyt |
| **B8R (20-27)** | TSYKFESV | **$K^b$** | 1,23 | 0,15 | dep | Luminal |
| **F1L (200-207)** | STREYLKL | **$K^b$** | 0,80 | 0,10 | dep | Cyt |
| **E7R (130-137)** | STLNFNNL | **$K^b$** | 0,77 | 0,10 | dep | Cyt |
| **F13L (307-315)** | FTIQNNTKL | **$D^b$** | 0,41 | 0,05 | ind | Cyt |
| **F5L (279-287)** | SAPMNVDNL | **$D^b$** | -0,44 | -0,05 | ind | 1 TM aa + 8 luminal aa |

**Table 9. MHC-I restricted epitopes included in Silvia Lázaro et al.**

The epitopes studied in our lab (Silvia Lázaro et al., in preparation) are summarized, tabulated according to their OHM score and their sequence, H-2 restriction, hydrophobicity score, TAP requirement(dep, dependent; ind, independent) and the location of the epitope within the protein is shown. TAP-ind epitopes are colored in grey. *Relative hydrophobicity is calculated by dividing the hydrophobicity score by the number of aa in the peptide sequence. TM: transmembrane; Cyt: Cytoplasmic; aa: aminoacid.

To make sure that any possible difference in hydrophobicity was not mediated by the hydrophobicity of the protein that the epitopes are derived from, we calculated in the same way the OHM score for all 30 VACV proteins included in the analysis. To be able to compare the scores of the proteins and the epitopes, we represented the relative hydrophobicity (OHM score/number of residues of the protein or peptide). The results show that the VACV proteins that include TAP-ind or TAP-dep epitopes do not differ in their hydrophobicity score, while the epitopes do. In harmony with Lautscham et al., TAP-ind epitopes showed higher hydrophobicity score than TAP-dep epitopes (**Figure 30**). Filtering the epitopes by their H-2 restriction, we observed that the difference in hydrophobicity between H-2K$^b$ restricted TAP-ind epitopes and H-2K$^b$ restricted TAP-dep epitopes is more significant than if we include also the H-2D$^b$ restricted epitopes.

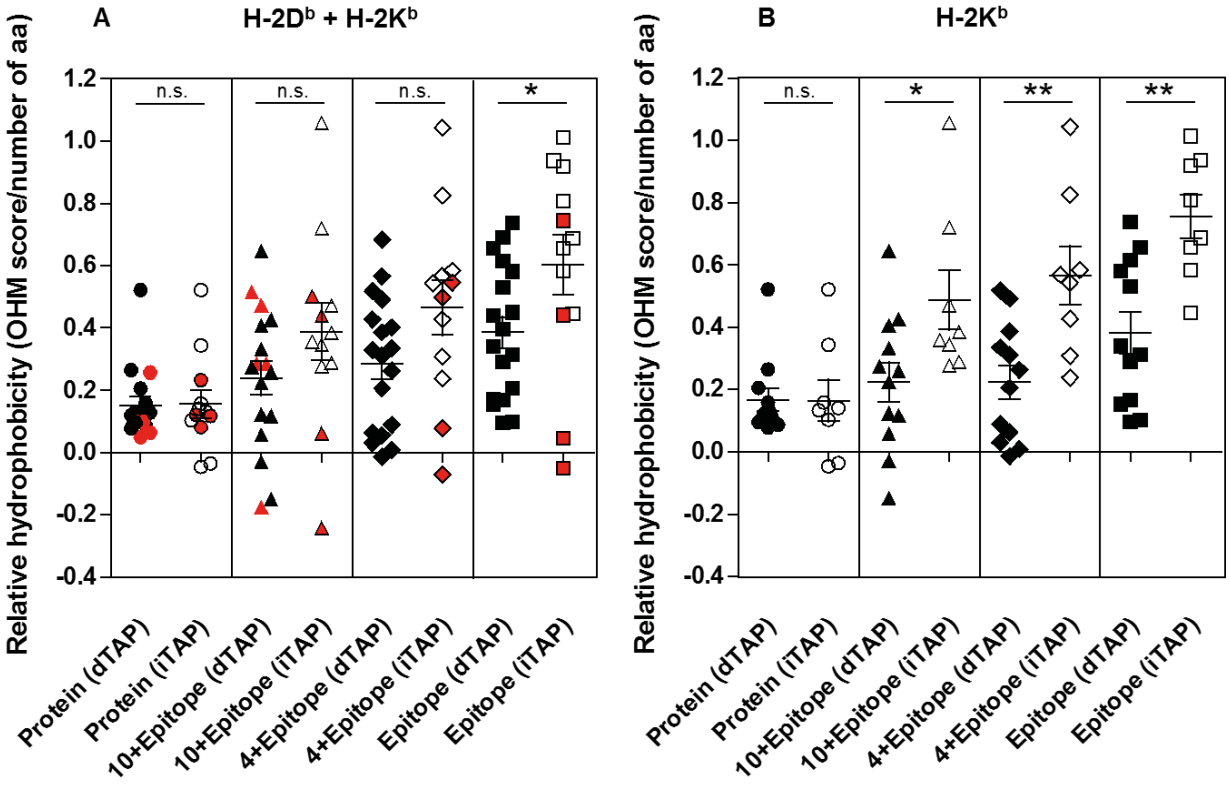



**Figure 30. TAP-independent epitopes are more hydrophobic than TAP-dependent epitopes, while the proteins they derive from show similar hydrophobicity scores.**

OHM hydrophobicity score was calculated for 30 immunogenic VACV-encoded peptides, precursors of these peptides (with 10 or 4 aa N-terminal extensions) and the proteins they derive from. H-2K$^b$ binding epitopes are depicted in black together with H-2D$^b$ binding peptides, depicted in red (**A**). All twenty H-2K$^b$ binding epitopes are graphed alone in **B**. Mean and SEM values for each group are shown.

### 5.4.2. Hydrophobicity of potential precursors of immunogenic VACV peptides.

Knowing that TAP-independent VACV MHC-I restricted epitopes are more hydrophobic than TAP-dependent epitopes and that the proteins they come from do not differ in this feature, we wondered how far this significant difference would be observed if we calculated the OHM score for different extensions of the epitopes. This would give some hints about which is the molecule transported in the absence of TAP, whether a precursor peptide or the ligand itself, assuming that hydrophobicity is the feature or one of the features that provide peptides with the capability to be transported in a TAP-independent manner. We calculated the OHM score for sequences including the epitope and 0 to 20 aa extended in the N-terminus, C-terminus or both for all 12 TAP-independent epitopes and 18 TAP-dependent epitopes. T-tests were performed to test the difference of means in hydrophobicity between TAP-ind and TAP-dep epitopes for each number of aa extended (**Figure 31**).

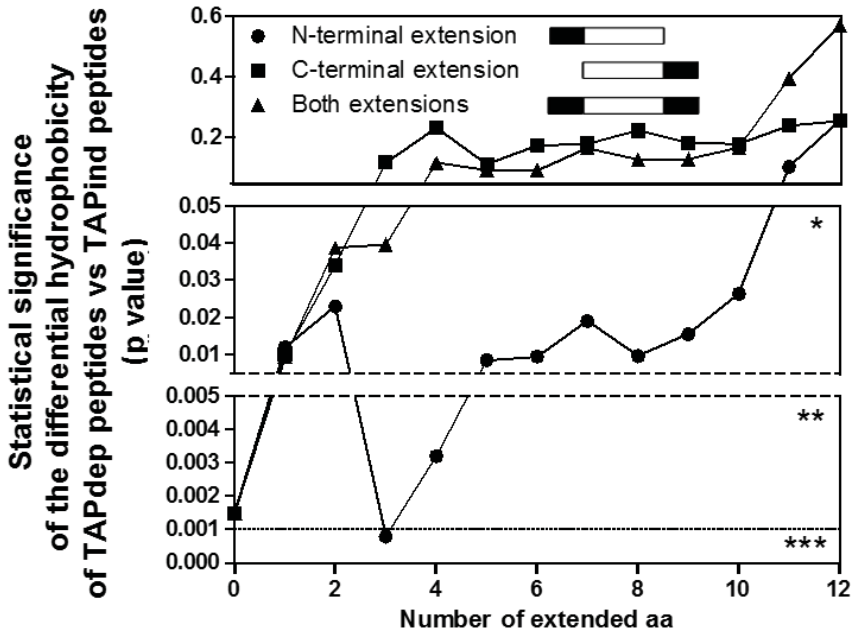



**Figure 31. Residues in the N-terminal extensions are differentially more hydrophobic in TAP-ind-processed epitopes than C-terminal residues.**

The OHM hydrophobicity score was calculated for the 20 VACV epitopes presented by the H-2K$^b$ haplotype previously studied and their potential precursors extended in N-terminus, C-terminus, or both. T-test analyses to compare the difference of means between TAP-ind and TAP-dep epitopes were performed. P values resulting from the tests for sequences including the epitope and each number of residues extended are shown (dotted line, p value=0.001; dashed line, p value=0.005; full line, p value=0.05). Extensions larger than shown resulted in less significant difference of hydrophobicity.

The results show that, while in C-terminal extended precursors the significant difference in hydrophobicity between TAP-ind and TAP-dep epitopes was rapidly lost, adding aa in the N-terminus preserved the differences for more than ten extended aa. That means that a precursor of a TAP-independent epitope extended up to ten aa in the N-terminus is very likely more hydrophobic than a similar precursor of a TAP-dependent epitope. Still assuming that hydrophobicity is indeed a relevant parameter in TAP-independent transport, these results would imply that the peptide itself or an N-terminally extended short precursor are the molecules transported to a MHC-I containing compartment in the absence of TAP.

Further, we calculated OHM scores for epitopes extended in different lengths at both the C- and N-termini. We studied if extensions at the C-terminus affect the differential hydrophobicity of N-terminally extended epitopes. The results show that extending aa at the C-terminus greatly affects the differential hydrophobicity of TAP-ind and TAP-dep peptides, and that N-terminally extended TAP-ind peptides are more distinguishable from N-terminally extended TAP-dep peptides when the extension is short (up to four residues) (**Table 10**).

| | | C-terminal extended aminoacids | | | |
|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 |
| N-terminal extended aminoacids | 0 | 0.0015 | 0.0100 | 0.0340 | 0.1179 |
| | 1 | 0.0120 | 0.0096 | 0.0344 | 0.1065 |
| | 2 | 0.0230 | 0.0166 | 0.0383 | 0.1311 |
| | 3 | **0.0008** | 0.0056 | 0.0146 | 0.0408 |
| | 4 | 0.0032 | 0.0178 | 0.0356 | 0.0736 |
| | 5 | 0.0085 | 0.3030 | 0.5030 | 0.1048 |
| | 6 | 0.0095 | 0.0299 | 0.0461 | 0.0922 |
| | 7 | 0.0191 | 0.0563 | 0.0861 | 0.1474 |
| | 8 | 0.0097 | 0.0295 | 0.0440 | 0.0800 |
| | 9 | 0.0156 | 0.0414 | 0.0598 | 0.1064 |
| | 10 | 0.0264 | 0.0639 | 0.0895 | 0.1440 |
| | 11 | 0.1057 | 0.1930 | 0.2537 | 0.3664 |
| | 12 | 0.2578 | 0.3863 | 0.4581 | 0.6129 |

**Table 10. The difference in hydrophobicity between TAP-ind and TAP-dep epitopes is more significant in short N-terminal-extended precursors.**

The OHM hydrophobicity score was calculated for the 20 immunogenic VACV epitopes presented by the H-2K$^b$ haplotype previously studied and their extensions in C and N-termini. T-student tests were performed to compare the difference in hydrophobicity between TAP-ind and TAP-dep epitopes and their extensions. The p values for each test are summarized in the table. Non-significant p values are non-colored, p values<0.05 are colored in light grey, p values between 0.05 and 0.005 are colored in grey and p values<0.001 are colored in dark grey.

### 5.4.3. Amphipaticity of epitopes and N-terminal extended precursors.

Further, we wondered if another peptide feature could also be potentially a characteristic to distinguish TAP-ind from TAP-dep epitopes. Amphipaticity is the organization of polar and non-polar residues of a helical peptide at different sides of the helix. It is calculated assuming a perfect helical conformation, calculating the vectorial sum of the hydrophobic moment of each aa. We speculated that the higher the amphipaticity of a peptide is, the easier it might pass through membranes (and therefore, bypass TAP). We calculated the amphipaticity of all 18 TAP-dep epitopes and 12 TAP-ind epitopes known in VACV and their N-terminal extended precursors, as these precursors may be the molecule transported through membranes according to hydrophobicity analysis. The results show, unlike hydrophobicity, no difference between TAP-dep and TAP-ind peptides, neither between the epitopes, nor between short N-terminal extended precursors (**Figure 32**).

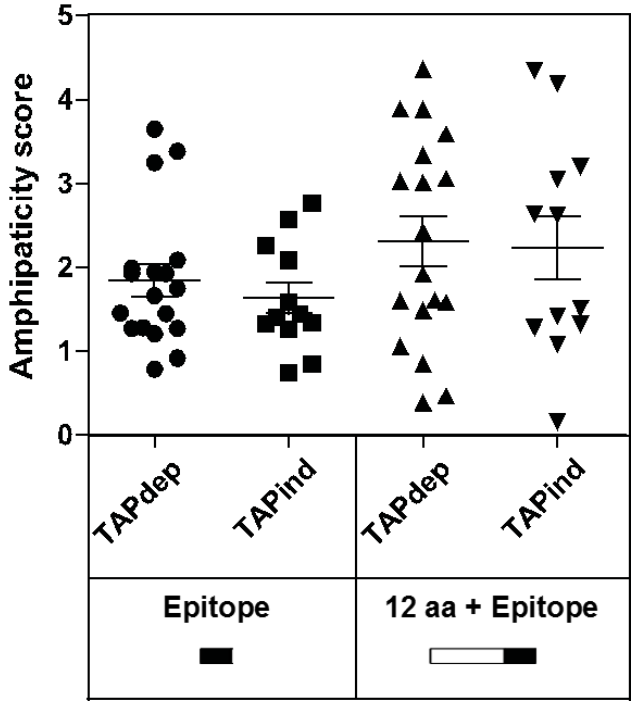



**Figure 32. Neither TAP-ind nor TAP-dep epitopes nor their N-terminal extended precursors differ in amphipaticity.**

Amphipaticity scores for 30 immunogenic VACV epitopes and their N-terminal extended precursors were calculated using the site "helical wheel projection" created by Don Armstrong and Raphael Zidovetzki: Version Id: wheel.pl,v 1.4 2009-10-20 21:23:36 don Exp. T-tests to compare difference of means were performed and none resulted statistically significant.

# 6. DISCUSSION.

## 6.1. Potential pathways for TAP-independent cross-presentation by BMDC.

To analyze the pathway or pathways involved in TAP-independent cross-presentation by BMDC we targeted the SNARE protein Sec22b, involved in the trafficking of vesicles from the ER to Golgi vesicles (Hay *et al.*, 1997; Zhang *et al.*, 1999) and to phagosomes (Becker *et al.*, 2005). By fusing ER-derived vesicles to the Golgi apparatus, Sec22b allows the anterograde transport of proteins from the ER, including loaded MHC-I molecules (**Figure 33A**). On the other hand, Sec22b allows the fusion of ER-derived vesicles to phagosomes, providing also the latter with components of the peptide loading complex (PLC) and making possible the load in phagosomes of MHC-I molecules with their ligands (**Figure 33B**).

The exact mechanism by which antigens are cross-presented in TAP-deficient BMDC is unknown. Knowing that TAP-independent cross-presentation of ovalbumin depends on proteasomal activity to take place in BMDC (Merzougui *et al.*, 2011), we proposed three potential pathways for this kind of antigen presentation. First, the antigen would access the ER in a TAP-independent way and load onto nascent MHC-I molecules, following the canonical antigen presentation route from that point (**Figure 33A**). A second possibility is that the antigen would access phagosomes in a TAP-independent way, binding MHC-I molecules coming from the ER to the phagosomes via Sec22b (**Figure 33B**). In a third alternative, the antigen would access phagosomes in a TAP-independent way and it would bind MHC-I molecules coming from the recycling of surface MHC-I (**Figure 33C**). Since the two first hypotheses depend on the function of Sec22b, we decided to silence its expression and assess the remaining cross-presentation in $TAP1^{-/-}$BMDC. Since the results showed that TAP-independent cross-presentation is completely independent of this protein (**Figure 9**), the third hypothesis gained more credibility. Also, the treatment with primaquine, which partially inhibits the recycling of MHC-I molecules (Reid and Watts, 1990; Houot *et al.*, 2015) increases the MHC-I molecule amount in endosomes in $TAP1^{-/-}$ BMDC, correlating with an increased antigen cross-presentation by the same cells under primaquine treatment (Merzougui *et al.*, 2011). This supports an important role of recycling endosomes in TAP-independent cross-presentation, as our data suggest. Taking together that (1) the proteasome is required for TAP-independent cross-presentation, (2) Sec22b is dispensable for OVA cross-presentation by $TAP1^{-/-}$ BMDC and (3) recycling endosomes play an important role in TAP-independent cross-presentation; we propose that after phagocytosis, the antigen would access the cytosol to be processed by proteasomes. After that, the proteasomal products, as it has been already proposed (Merzougui *et al.*, 2011), would access

phagosomes in a TAP-independent way where they would bind MHC-I molecules coming from recycling endosomes and finally, the MHC-I/peptide complex would be presented back on the plasma membrane (**Figure 33C**).

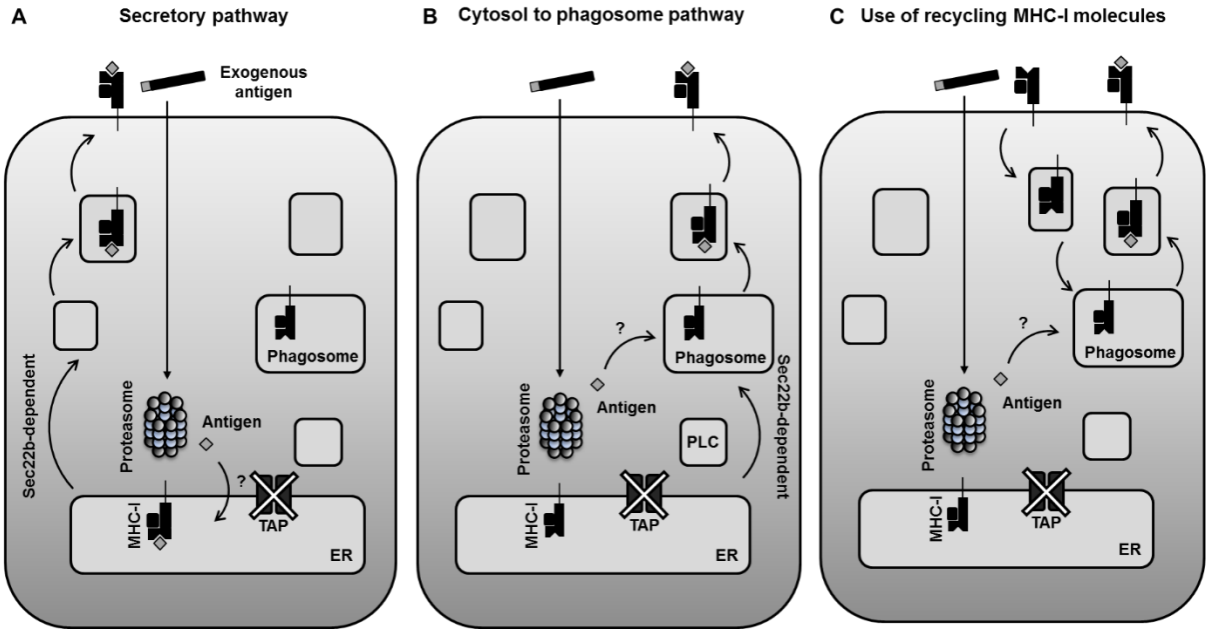



**Figure 33. Diagram showing the potential pathways for TAP-independent cross-presentation.**

The diagram shows our three different hypotheses for TAP-ind antigen cross-presentation. In **A**, the processed antigen would access the ER through an unknown mechanism and would follow the canonical secretion pathway to be loaded onto MHC-I in a Sec22b-dependent process. In **B**, the processed antigen would access the phagosome from the cytosol by an unknown mechanism and would be loaded in the phagosome onto MHC-I molecules coming from the ER in a Sec22b-dependent manner. In **C**, the processed antigen would access the phagosome from the cytosol by an unknown mechanism and would be loaded onto recycled MHC-I molecules from the plasma membrane in the phagosome.

On the other hand, cross-presentation of the same antigen by C57BL/6 BMDC does require the function of Sec22b to some extent (Cebrian *et al.*, 2011) and we have reproduced these results. Most probably, the internalized exogenous antigen follows the canonical antigen presentation route after processing by proteasomes. In fact, the remaining antigen presentation after silencing Sec22b in C57BL/6 BMDC is very similar to that of TAP1$^{-/-}$ BMDC (**Figure 9**). This suggests the existence of at least two paths of cross-presentation of OVA-beads by BMDC: one dependent on TAP and Sec22b, and other independent of both TAP and Sec22b (**Figure 34**).

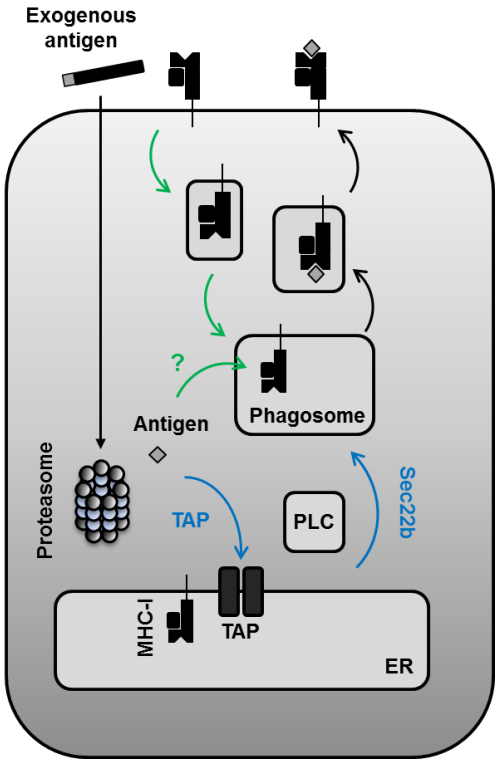



**Figure 34. Diagram showing the main hypothesis for TAP-dependent and TAP-independent antigen cross-presentation by BMDC.**

After internalization of the full length antigen to the dendritic cell and export to the cytosol from phagosomes for proteasomal cleavage, the processed antigen would access the phagosome either by TAP (blue) or bypassing this transporter (green). There, the antigen would load either onto MHC-I molecules coming from the ER via Sec22b (blue) or onto MHC-I molecules coming from the plasma membrane (green).

## 6.2. Alternative routes of direct MHC-I viral antigen presentation.

Alternative routes of direct MHC-I antigen presentation refer to all routes that bypass one or more of the key players in canonical direct presentation, such as (1) proteasomes, (2) TAP or (3) nascent MHC-I molecules. For some time, it was assumed that most TAP-independent antigens were derived from proteins entering the ER through their signal sequence and therefore, besides not needing TAP, they would also not require proteasomal cleavage (reviewed in (Del Val *et al.*, 2011)). However, several cases of antigens processed in a TAP-independent but proteasome-dependent way have been coming out (Lautscham *et al.*, 2001; Merzougui *et al.*, 2011; Oliveira *et al.*, 2014), breaking the old barrier between canonical and non-canonical antigen processing pathways.

### 6.2.1. Proteolytic enzymes involved in TAP-independent antigen presentation pathways.

Proteolytic enzymes catalyze the cleavage of peptide bonds, either in proteins or peptides. Their functions are varied, from maintaining the cellular homeostasis to producing a functional form of a protein. They also play a key role in antigen processing, making possible the cleavage and trimming of proteins and peptides, producing a huge amount of products differing in sequence as well as in length.

While proteasomes have had a key role in antigen processing, the generation of MHC-I ligands is not limited to this protease, but there is an increasing number or proteases also involved in antigen processing (reviewed in (Lázaro *et al.*, 2015)). They can trim proteasomal products (Stoltze *et al.*, 2000; Saric *et al.*, 2001; Towne *et al.*, 2005; Towne *et al.*, 2008; Zervoudi *et al.*, 2013; Gonzalez-Villalobos *et al.*, 2013) but they can also work independently of proteasomes (Gil-Torregrosa *et al.*, 1998; Tomkinson, 1999; van Endert, 2008; López *et al.*, 2010; Parmentier *et al.*, 2010; Oliveira *et al.*, 2013).

One of the aims of this work was to assess the role of proteasomes and other proteases in TAP-independent MHC-I viral antigen presentation. While the background in TAP-ind antigen presentation assumed that TAP-independency meant independence of proteasome cleavage, it has been suggested that that observation may respond to a bias in the research of TAP-ind antigens (Del Val *et al.*, 2013). The results we obtained show, on the other hand, that TAP-independency does not necessarily mean proteasome-independency, yet in agreement with other similar study (Oliveira *et al.*, 2014). In fact, proteasomal cleavage is highly important in the generation of TAP-independent MHC-I VACV antigens (**Figure 16B and C**), even to the same extent as in global MHC-I VACV antigen presentation (**Figure 17**).It is true that, as the transcription of post-replicative expression genes is inhibited by the proteasome inhibitor lactacystin (Satheshkumar *et al.*, 2009), we could be overestimating the effect of lactacystin on VACV TAP-ind antigen presentation, but it is also known that around 65% of the CD8+ T lymphocytes activated against VACV epitopes are specific for early expression genes (Moutaftsi *et al.*, 2010).As proteasomes are the major and more multicatalytic cellular proteases, this would imply that both TAP-dep and TAP-ind pathways sample undistinguishable intracellular pools of peptides for proteolytic action. Interestingly, the fraction of antigenic VACV proteins located in cytosol (compared to those in vesicular compartments) is similar for TAP-dep and TAP-ind routes, as well as for $LC^R$ and $LC^S$ pathways (Lázaro S, PhD report).The remaining antigen presentation in $TAP1^{-/-}$ BMDC that we detected after LC treatment (33% of TAP-independent viral antigen presentation and 6.6% of global viral antigen presentation) could be due either to an incomplete inhibition of proteasomal cleavage by the inhibitor LC or else to antigens generated in a TAP-independent and LC-resistant way. Also, the inhibition of post-replicative viral genes replication by the treatment with LC could be underestimating the possible $LC^R$ TAP-dep and $LC^R$TAP-ind viral antigen presentation. We generated specific CTL lines against TAP-independent VACV epitopes to find out if some of them could actually be generated in the presence of LC. We found that three out of six epitopes tested were LC-resistant (A19L, A6L and A25L)in C57BL/6 BMDC (**Figure 20**). In addition, we could find an alternative proteolytic pathway for one of them (A25L).The generation of this epitope was highly impaired in the

presence of dec-RVKR-cmk, suggesting an important role of pro-protein convertases. (**Figure 24**). This family of proteases was already known to be involved in antigen presentation (Gil-Torregrosa *et al.*, 1998; Gil-Torregrosa *et al.*, 2000; Medina *et al.*, 2009). However, the failure in finding alternative proteases for the generation of the epitopes A19L and A6L in the presence of LC shows that there are other proteases besides those included in this work that are responsible for their generation.

The global analysis of the epitopes studied in our lab (**Table 5**) shows a non-preferential cleavage by proteasomes of TAP-dependent and TAP-independent epitopes, supporting the idea that proteasomal cleavage and TAP transport are not necessarily linked. In a scenario in which proteolytic enzymes and transport through membranes work separately in the generation of antigens, the possibilities in the generation of a given antigen are quite open. This would establish that there is not a sole alternative TAP-independent pathway, but many.

Overall, we must not forget that the techniques here used have a good but lower sensitivity to detect MHC-I/peptide complexes than mass spectrometry-based approaches (Purcell *et al.*, 2016). This could underestimate to some extent the level of antigen presentation we are detecting in this work.

## 6.3. Potential mechanisms and products for TAP-independent transport.

While we know increasingly more epitopes that are presented in a TAP-independent way (Gueguen *et al.*, 1994; Gil-Torregrosa *et al.*, 1998; Sigal and Rock, 2000; Lautscham *et al.*, 2001; Oliveira *et al.*, 2011),the mechanisms and routes that make possible the transport of peptides through membranes in a TAP-deficient scenario are largely unknown. None of the studies describing TAP-independent antigen presentation have succeeded in finding a defined pathway or mechanism that can transport those antigens through membranes other than TAP. Even more, we do not know what makes an antigen suitable for TAP-independent transport or which exact molecule is transported in a TAP-independent way. Here, we discuss the differential characteristics found in TAP-dependent and TAP-independent antigens and the possible and more likely protein products that are transported in the absence of TAP.

### 6.3.1. Differential characteristics between TAP-independent and TAP-dependent antigens.

In the seek of a common characteristic in TAP-independent antigens that can also differentiate them from TAP-dependent peptides, only a few studies have succeeded in

finding one ((Lautscham *et al.*, 2003) and Lázaro S. et al., in preparation). Many characteristics of the peptides and the proteins they derive from have been analyzed, such as temporal expression (in the case of viral proteins), protein function, protein localization, localization of the epitope within the protein, net charge of peptides and their precursors (Lázaro S. et al, inpreparation) and hydrophobicity of proteins, precursors (**Figure 30** and **Figure 31**) and peptides ((Lautscham *et al.*, 2003) and Lázaro S. et al.); as well as amphipaticity of precursors and peptides (**Figure 32**). However, besides minor variations in MHC-I bindng motifs, only peptide and precursor hydrophobicity has been determined to be significantly different in TAP-independent peptides when compared with TAP-dependent peptides. Also, a higher prevalence of peptides derived from viral structural proteins for TAP-independent epitopes than in their TAP-dependent counterparts was found (Lázaro S. et al, in preparation).

But, if higher hydrophobicity is indeed allowing peptides to be transported in the absence of TAP, which could be the mechanisms responsible? As reviewed(Del Val *et al.*, 2011), there are different mechanisms that could be capable of transporting a peptide or protein through membranes:

1. Co-translational transport through Sec61 of membrane and secreted proteins.
2. Transport to the ER by another transporter.
3. Transport to endolysosomal compartments by another transporter.
4. Transport to endolysosomal compartments by autophagy.
5. Diffusion through membranes.
6. Post-translational transport of small peptides, including cleaved signal sequences, by calmodulin (Shao and Hegde, 2011).
7. Post-translational transport to the Sec61 translocon of tail-anchored and short secretory proteins by TRC40/WRB (Yamamoto and Sakisaka, 2012; Johnson *et al.*, 2012).

Of all these potential mechanisms, hydrophobicity would only benefit transport by another transporter with high affinity for hydrophobic peptides and also could lead to passive diffusion through membranes (2, 3, 5, 6 and 7). However, although in this work we failed in silencing the expression of the peptide transporter TAPL to assess its possible role in MHC-I antigen presentation, other authors (Oliveira *et al.*, 2014) could observe that silencing this gene did not affect at all the presentation of a TAP-independent self peptide. On the other hand, there are no studies relating passive diffusion through membranes and TAP-independence.

### 6.3.2. Determination of the length of protein products that are transported in the absence of TAP.

In order to find one or more TAP-independent antigen transport pathways we decided to focus on the potential substrates of transport. Once a protein is synthesized, an antigenic peptide derived from it could access a MHC-I-containing compartment by three different ways: (1) the protein itself accesses the compartment passing through the translocon (if it contains a signal sequence) or other post-translational translocation mechanism (if it doesn't contain a signal sequence) and the peptide is generated in the vesicular pathway (**Figure 35A**); (2) the protein, if cytosolic, is partially degraded in the cytosol and a precursor of the antigenic peptide is transported through membranes (**Figure 35B**) and(3) the cytosolic protein is partially degraded and the correct MHC-I ligand is transported through membranes to the vesicular pathway (**Figure 35C**).

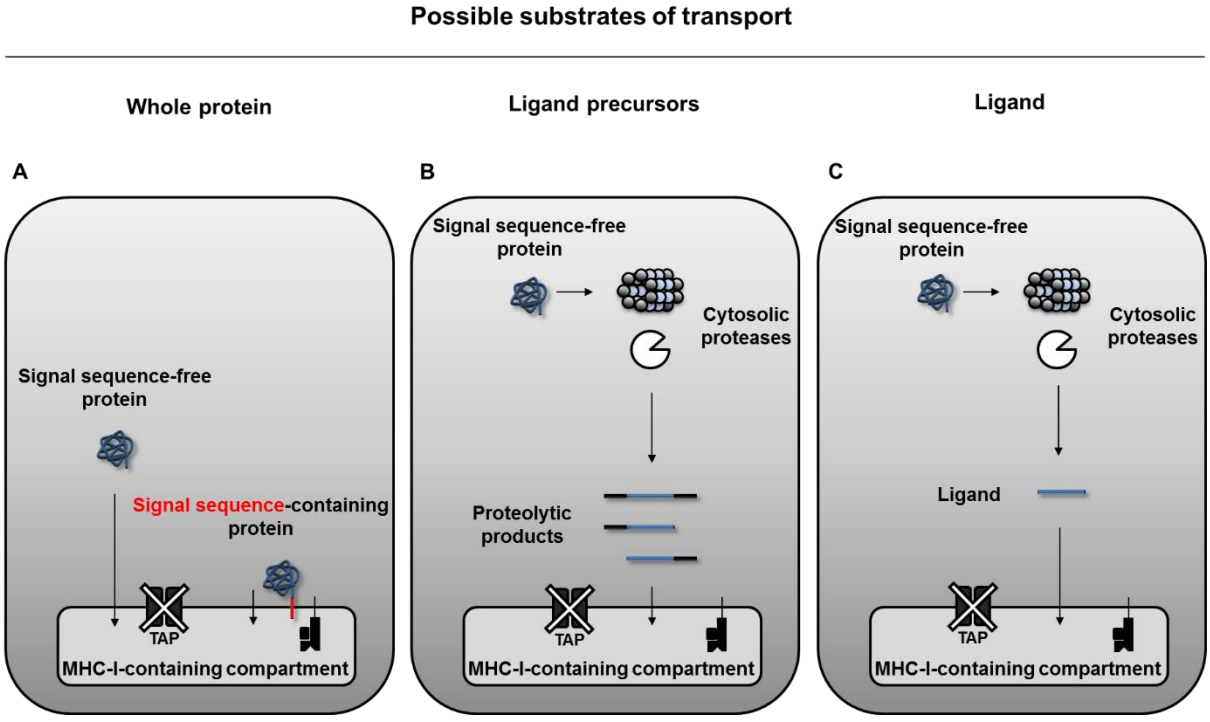



**Figure 35. Potential substrates of transport to MHC-I-containing compartments in the absence of TAP.**

The diagram shows the three possible cytosolic substrates for the transport to the ER. In **A**, the whole protein is transported to the MHC-I-containing compartment, either mediated by a signal sequence or other mechanisms. In **B**, the protein is partially cleaved by cytosolic proteases and the ligand precursors are transported to the MHC-I-containing compartment. Finally, in **C** the cytosolic proteases cleave the parental protein and the product is the final ligand which is transported to the MHC-I-containing compartment and is loaded onto MHC-I molecules.

We considered all three possibilities to determine as accurately as possible the most likely antigenic substrates of transport through membranes in the absence of TAP.

In the first hypothesis, the protein itself can be transported through membranes in the absence of TAP. That would require that the proteins that harbor TAP-independent antigens share a common pattern or characteristic that allows them to be transported in a TAP-independent way. As mentioned in Introduction, the study of the characteristics of twelve VACV proteins that harbor twelve TAP-independent peptides show that there is not a common link among them that differentiates them from proteins that do not harbor TAP-independent antigens. Also, the fact that there are two VACV proteins each of them harboring a TAP-independent antigen and a TAP-dependent antigen at least indicates that a common characteristic among proteins harboring TAP-ind peptides cannot be the general rule for all of them. The hypothesis that the proteins access the vesicular pathway and they are entirely cleaved there to generate antigenic peptides is probably wrong, as most of VACV MHC-I-restricted TAP-ind antigen presentation requires the action of proteasomes (**Figure 16B**and**Figure 17**).

That leaves two possible transport substrates: the antigenic peptide itself or a longer precursor. Our results show that TAP-independent antigenic peptides are more hydrophobic than TAP-dependent, but this differential hydrophobicity is also maintained in longer precursors, more markedly and farther for N-terminally elongated precursors (**Figure 31**). Thus, TAP-independent VACV antigens are strongly dependent on proteasomal action and TAP-independent VACV peptides as well as N-terminally elongated TAP-independent precursors are more hydrophobic than their TAP-dependent counterparts. With these data we propose a model of TAP-independent antigen processing and transport (**Figure 36**) in which proteasomes, possibly together with other cytosolic proteases, would cleave proteins in the cytosol and generate the final C-terminus of the MHC-I ligand. Highly hydrophobic N-terminally extended proteasomal products would then be transported through membranes in the absence of TAP. The N-terminal extension would then be removed in the vesicular pathway by aminopeptidases (like ERAAP) to generate the correct MHC-I ligand, that would bind MHC-I molecules and be transported to the plasma membrane (**Figure 36**). Participation of luminal carboxypeptidases such as ACE in the TAP-ind pathway cannot be excluded but our analysis suggests that they would play a minor role. In fact, other similar study shows the importance of both proteasomes and metalloaminopeptidases in the generation of a TAP-independent cellular antigen (Oliveira *et al.*, 2014), which agrees with our data on viral antigens (**Figure 24A**).

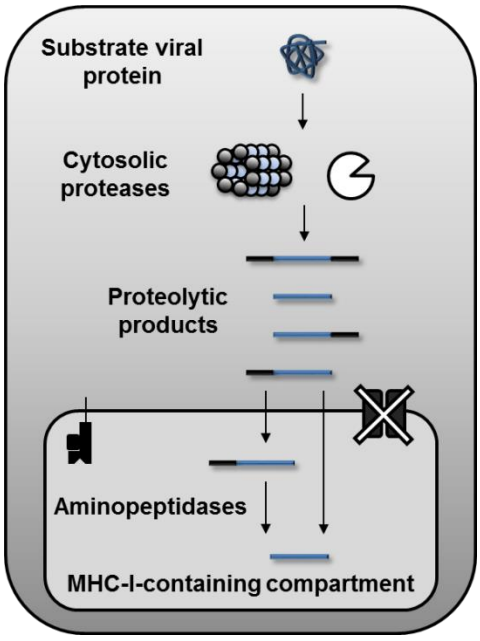



**Figure 36. Model of TAP-independent antigen processing and transport.**

Our final model of TAP-independent viral antigen processing involves endo- and carboxypeptidase action in the cytosol by proteasomes and endo- and aminopeptidase action by and other proteases. Higher hydrophobicity of peptides would lead to the recognition and transport to MHC-I-containing compartments of N-terminally-extended precursors or the ligands themselves. N-terminally-extended precursors would be trimmed by aminopeptidases to generate the final ligand.

## 6.4. Relevance of studying TAP-independent antigen processing pathways for vaccination and immunotherapy.

### 6.4.1. TAP-independent pathways in the prevention of viral infections. Possibilities and limitations.

$CD8^+$ T lymphocytes need to find their specific antigen presented on MHC-I molecules on a cell surface to perform their cytolytic action. Most of the antigens need the transport to the ER by TAP to be properly processed and presented. However, we have demonstrated the unexpected prevalence of TAP-independent viral antigen presentation pathways (Lázaro S. et al, in preparation). But, are these TAP-independent antigen presentation pathways relevant for the immune system in order to clear a viral infection *in vivo*?

TAP-deficient patients, although they suffer from recurrent bacterial infections, can control viral infections as efficiently as healthy patients (Gadola *et al.*, 2000). In harmony with that observation, our results show that $TAP1^{-/-}$ mice can clear a VACV WR infection as efficiently as C57BL/6 mice (**Figure 25**). Hence, the deficiency in TAP does not lead to a complete shutdown of the immune system against viral infections. In particular, $CD8^+$ T lymphocytes can also control a VACV WR infection in mice that lack TAP when they are previously primed with a TAP-independent epitope in the absence of priming of any other immune mechanism (**Figure 27**). Although $CD8^+$ T lymphocyte response is enhanced for the TAP-ind epitope J3R after BMDC/peptide vaccination and VACV challenge, this does not occur for the TAP-ind epitopes A19L and A25L in $TAP^{-/-}$mice (**Figure 28**). It is known that

antigen can affect the immune response, and this correlation is not lineal, but reaches a plateau, different for each epitope (Wherry *et al.*, 1999; Tscharke *et al.*, 2015). It may be happening that for J3R the VACV dose we used was sufficient to trigger a detectable production of IFNγ, while it was insufficient for the epitopes A19L and A25L.

However, our results also show a defect in viral clearance by $CD8^+$ T lymphocytes in ovaries (in contraposition with spleens) when mice lack TAP (**Figure 26**). This differential capability of viral clearance by $CD8^+$ T lymphocytes in the absence of TAP in different organs shows limitations for vaccination in the absence of TAP that need to be solved in order to develop efficient vaccines in TAP-deficient pathologies (TAP-deficient patients, tumors silencing TAP and viruses blocking TAP).Since this defect cannot be explained by a lower expression of MHC-I in $TAP1^{-/-}$ ovaries than in C57BL/6 ovaries (**Figure 29B**) and the epitopes assayed in ovaries were protective in spleens, it seems that there is no general limitation from the TAP-deficient presenting cell, unless there are tissue-specific proteases in different organs that limit the presentation of certain TAP-ind epitopes.

By producing reactive oxygen and nitrogen species, neutrophils work together with $CD8^+$ T lymphocytes to clear VACV in mice (Hickman *et al.*, 2013). Hence, we could explain this differential antiviral $CD8^+$ T lymphocyte efficiency between organs by a less effective infiltration of neutrophils in ovaries than in the spleen, leaving the $CD8^+$ T lymphocyte action much less effective in ovaries (with less neutrophils) than in the spleen (with more neutrophils). It is known that the level of neutrophil infiltration in ovaries depend on the estrous cycle stage (Sasaki *et al.*, 2011), and the estrous cycle itself is driven by a light/dark cycle (Miller *et al.*, 2004). Since in our experiments we did not take into account the estrous cycle stage, we cannot assure the level of neutrophil infiltration in the ovaries at the time of the infection. Experiments detecting the level of neutrophil infiltration in spleen and ovaries in $TAP1^{-/-}$ and C57BL/6 mice under a VACV infection would need to be done to address this issue.

It is also known that more than one contact between a CTL and an MCMV-infected cells is needed for the correct elimination of the target infected cell, being 3 contacts sufficient to extremely raise the probability of this elimination (Halle *et al.*, 2016) (**Figure 37**). Our results show that ovaries, independently of the presence or absence of TAP, harbor $10^5$ more viruses than spleens in similar days post-infection (**Figure 29A**). Uninfected $TAP1^{-/-}$ mice have between 8-30 times less $CD8^+$ T lymphocytes than C57BL/6 mice in spleen, blood and lymph nodes (van Kaer *et al.*, 1992). The data of the VACV-infected mice included in our experiments reveal10 times less $CD8^+$ T lymphocytes in $TAP1^{-/-}$spleens compared to C57BL/6 spleens and 4 times less $CD8^+$ T lymphocytes in $TAP1^{-/-}$PEC than in C57BL/6 PEC.

Also, it has been proposed that CTL response is not only determined by the number of CTL precursors for a given epitope, but also for the number of CTL precursors above a minimal affinity/avidity threshold (Tscharke *et al.*, 2015), an unknown data for us. Therefore, we can assume that in TAP1$^{-/-}$mice the contacts between infected cells and CTL are less frequent, making it more difficult to clear enough infected cells. This would be especially difficult in highly infected ovaries, but not so critical in spleens, which contain fewer infected cells.

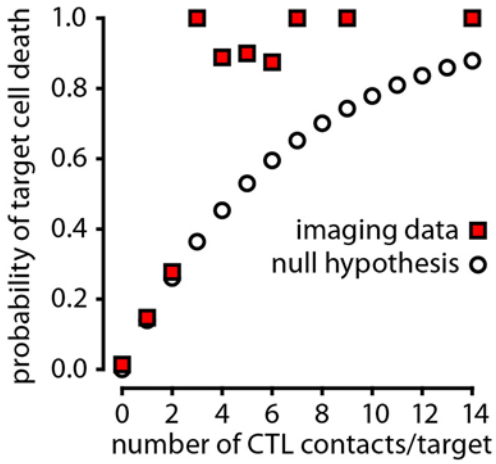



**Figure 37. Probability of target-cell death for infected cells contacted by 0–14 CTL.**
(Halle *et al.*, 2016)Data from two-photon *in vivo* imaging shows the probability of death of an MCMV-infected cell attending to imaging data (red) or the null hypothesis (every CTL contact is an independent event and previous contacts do not influence the target cell's death probability; white).

Another variable that might be interfering with these results is that the infection route determines the virus spread and therefore, the viral load in different organs. In addition, CD8-mediated specific cell lysis is higher when the organ is closer to the infection site (Lin *et al.*, 2013). Thus, our model of systemic intraperitoneal infection might be shaping the lytic capacity of CD8$^+$ T lymphocytes in ovaries and spleen.

All these hypotheses are not contradictory and could be working at the same time in our infection model. We would be in a scenario in which the absence of TAP does not impede the presentation of immunogenic and protective MHC-I-presented epitopes nor affect the functionality of the CTL themselves. The limitations in the elimination of viral infections in the absence of TAP would consist in (1) tissue-specific proteases that participate in the processing if these TAP-ind epitopes, (2) the synergic action of CTL and neutrophils and the efficiency for the latter to infiltrate in some tissues and/or (3) the ratio of CTL/infected cell, establishing an upper threshold of infected cells that restricts CD8$^+$ T lymphocytes from performing a proper viral clearance.

### 6.4.2. Interference of viral proteins with TAP.

It is widely known that some pathogens express proteins that interfere with elements of the antigen presentation machinery, lowering the direct presentation of antigens by the infected cells and therefore, lowering the cellular immune response (reviewed in (Mayerhofer

and Tampe, 2015)). TAP is one of the targets of these pathogens in their evolutionary struggle to attenuate the host immune system. In **Table 11** the pathogen molecules that block TAP function in the host discovered so far are summarized. Of note, there is one protein expressed by bacteria, suggesting an important role of TAP and MHC-I molecules in the response against bacteria. Five proteins out of seven are encoded by viruses from the Herpesviridae family. These viruses are very common in the human population. According to the World Health Organization, about two thirds of the population under 50 are infected with HSV-1 (Looker *et al.*, 2015a), HCMV prevalence in between 40-70 % of the adults (Lamberson and Dock, 1992). This number rises to 90 % during the first two decades of life of individuals worldwide for EBV (Ng and Khoury, 2009).

| Organism | | Protein | Mechanism of action | References |
|---|---|---|---|---|
| *Herpesviridae* | HSV-1 | **ICP47** | Binds to peptide-binding region of human TAP. | (Lacaille and Androlewicz, 1998) |
| | HCMV | **US6** | Binds from ER lumen to human TAP, changing to an ATP non-binding conformation. | (Hewitt *et al.*, 2001) |
| | Varicelloviruses (BHV/EHV/PRV/VZV) | **UL49.5** | Ubiquitination of TAP leading to degradation/inhibits ATP binding to TAP. | (Koppers-Lalic *et al.*, 2005)<br>(Koppers-Lalic *et al.*, 2008)<br>(Koppers-Lalic *et al.*, 2008) |
| | EBV | **BNLF2a** | Blocks ATP and peptide binding to TAP. | (Croft *et al.*, 2009) |
| | Murine γ-herpesvirus-68 | **MK3** | Ubiquitination of TAP leading to degradation. | (Boname *et al.*, 2005) |
| *Poxviridae* | CPXV | **CPXV12** | Inhibits peptide translocation by binding ATP site. | (Alzhanova *et al.*, 2009) |
| *Bacteria* | *Pseudomonas aeruginosa* | **Cif** | Ubiquitination of TAP leading to degradation. | (Bomberger *et al.*, 2014) |

**Table 11. TAP inhibitory molecules expressed by pathogens.**

Molecules expressed by pathogens that interfere with the function of TAP in the infected cell are summarized in this table. HSV-1: Herpes simplex virus 1; HCMV: Human cytomegalovirus; BHV: Bovine herpes virus; EHV: Equine herpes virus; PRV: Pseudorabies virus; EBV: Epstein-Barr virus; CPXV: Cowpox virus.

### 6.4.3. TAP-independent pathways in cancer immunotherapy.

The first treatments of cancer started after World War II, when it was observed that alkylating agents killed growing cancer cells by damaging their DNA. The first cancer was cured in 1956 with the compound methotrexate, which is still commonly used in chemotherapy. Along with radiotherapy, chemotherapy has been widely used to treat many types of cancer. However, since both approaches are very aggressive for healthy cells and cause many side effects to the patients, other approaches have been developed to treat cancer. With the study of the biology of cancer, the blocking of oncogene products has developed into very efficient cancer therapies. In addition, identification of tumor-specific cell surface antigens has led to their targeting by antibodies and to effective cancer therapies. For instance, the antibody trastuzumab against the human epidermal growth factor receptor 2 protein (HER2) was approved during the late 1990s to treat metastatic breast cancer (Goldenberg, 1999). The involvement and potential use of the natural immune response in the control of cancer cells was not noticed until the 2000s, when the first drugs that modulated the immune response were used. A good example is the anti-programmed cell death protein 1 (PD1) antibody nivolumab, which accomplished very promising results against solid tumors (Brahmer *et al.*, 2010; Topalian *et al.*, 2012) and nowadays is commonly used with very promising results.

The treatment of cancer by modulating the immune response is possible because the action of $CD8^+$ T lymphocytes is not limited to infected cells, but they can also recognize and kill malignant cells. As $CD8^+$ T lymphocytes patrol through the organism, they may encounter tumor cells presenting tumoral antigens on their surface, leading to subsequent elimination of the malignant cell, provided the tumor tissue microenvironment is not suppressing or regulating their action. However, tumors that interfere with the antigen presentation machinery are common. Particularly, 75 % of them silence TAP expression (36 out of 48)(Leone *et al.*, 2013a). In these tumors, MHC-I surface expression is greatly diminished and $CD8^+$T lymphocyte action is therefore limited. It is known that $CD8^+$ T lymphocytes can recognize epitopes that are presented by different tumoral TAP-deficient cell lines (Wolpert *et al.*, 1997; van Hall *et al.*, 2006). Most of the work in mice has been done with the TAP-deficient model RMA-S, which we show is less efficient in presenting antigens in the absence of TAP than primary cell cultures (**Figure 15**). This might be due to the fact that the

expression of the potential alternative transporter TAPL is diminished in RMA-S cells compared to their TAP-proficient counterpart RMA cells (**Figure 14**). Even so, many promising results have been achieved. Of note, the priming of WT mice with a peptide specific of TAP-deficient cells can prevent TAP-deficient tumor growth in these animals (van Hall *et al.*, 2006). This group was the first in describing a class of antigens that are only presented in cells lacking TAP: "T-cell epitopes associated with impaired peptide processing" (TEIPP). This characteristic makes these epitopes very interesting for an immunotherapeutic approach, since trained $CD8^+$T lymphocytes would only recognize and therefore eliminate cells lacking the transporter, leaving healthy cells untouched, unlike current treatments like chemotherapy or radiotherapy or oncogenic-specific inhibitors. This warrants safety in clinical trials and would solve the main problem of serious adverse effects of other treatments, making this new approach a potential substitution or complement of current tumor treatment.

Far from being an exception in some mouse model, CTL specific for antigens presented only by TAP-deficient cells have been found in healthy human donors (Lampen *et al.*, 2010), widely opening a window for immunotherapy of TAP-silenced human cancers.

Currently, several approaches to fight cancer with immunotherapy are being performed. The first cancer immunotherapy approach that has achieved good results are the immune checkpoint inhibitors. These inhibitors are monoclonal antibodies that block the function of proteins like PD-1 or CTLA-4 that negatively regulate the proliferation and activation of $CD8^+$ T lymphocytes. Thus, the blocking of these proteins unleashes the functionality of the $CD8^+$ T lymphocytes. This approach is normally combined with other kind of cancer treatments like radiotherapy or chemotherapy, improving the recovery of up to 30 % of the patients (Schumacher and Schreiber, 2015)

The underlying mechanism of this approach involves neoantigens. Neoantigens are antigens that arise in tumoral cells as a result of a mutation in a cellular protein. Therefore, CTL specific for one or more of these antigens would only clear tumoral cells, leaving healthy cells intact. The availability of different kinds of antigens that are exclusively expressed in tumoral cells provides us with another possibility to fight these malignant cells with our own immune system. However, there are limitations to this approach, since it is not known whether all tumors express neoantigens or TEIPPs, and the use of neoantigens in patients require the individual characterization of these antigens for every patient and the development of individual treatments, with the economic costs involved. In **Table 12** it is summarized the main advantages and disadvantages of neoantigens and TEIPPs in the immunotherapy of cancer.

| | Antigens prefentially expressed by tumors | Neoantigens | TEIPPs |
|---|---|---|---|
| **Advantages** | Certain level of selectivity. Substitution of more aggressive therapies. | Useful for the tumors with a high rate of mutation. | TEIPPs can be common in the same kind of tumors in different patients. |
| **Disadvantages** | Non-complete selectivity. | Distinct in every patient. Need to develop individual treatments. | Limited to tumors that silence TAP. |

**Table 12. Advantages and disadvantages of neoantigens and TEIPPs in cancer immunotherapy.**

However, very few TEIPPs are described. The treatment of TAP-silenced tumors would be much eased by a database of TEIPPs for each human HLA. Therefore, the same kind of tumors in different patients could be treated just by characterizing the HLA expressed in each patient. Thus, the study and characterization of these epitopes could highly improve the treatment of some human cancers. Of note, TEIPPs presentation is restricted to cells that do not express TAP but do express the rest of molecules involved in the antigen presentation machinery, such as MHC-I molecules. The treatment of patients targeting TEIPPs would then be restricted to tumors with these characteristics.

The high prevalence of viral infections and tumors that can block TAP function shows the importance of researching on TAP-independent antigen presentation in the seek for effective and safe vaccines. Characterizing TAP-independent viral antigens can lead us to find more easily and more rapidly proper candidates for vaccines to fight pathogens and cancers that can block TAP function. Also, finding common characteristics among TAP-independent antigens that differentiate them from TAP-dependent antigens could help us predict TAP-independent epitopes in newly found pathogens that could block TAP as well as in tumors.

## 7. CONCLUSIONS.

1. TAP-independent cross-presentation of OVA-coated beads does not require the SNARE protein Sec22b function, unlike TAP-dependent OVA-coated beads cross-presentation.

2. Proteasomes participate to the same extent in the processing of VACV TAP-dependent and TAP-independent epitopes.

3. Pro-protein convertases participate in the processing of at least one VACV epitope in both TAP-dependent and TAP-independent pathways.

4. TAP1$^{-/-}$ mice clear VACV from spleens and ovaries *in vivo* as efficiently as C57BL/6 mice.

5. Vaccination of TAP1$^{-/-}$and C57BL/6 mice with VACV TAP-independent epitopes boosts CD8$^{+}$ T lymphocyte-mediated anti-viral effect.

6. VACV TAP-independent epitopes and N-terminally extended precursors are significantly more hydrophobic than their VACV TAP-dependent counterparts.

## 7. CONCLUSIONES.

1. La presentación cruzada independiente de TAP de bolitas recubiertas de OVA no requiere de la función de la proteína SNARE Sec22b, al contrario que su presentación cruzada TAP-dependiente.

2. Los proteasomas participan de la misma manera en el procesamiento de epítopos de VACV TAP-dependientes y TAP-independientes.

3. Las pro-protein convertasas participan en el procesamiento de al menos un epítopo de VACV tanto en su vía TAP-dependiente como TAP-independiente.

4. Los ratones TAP1$^{-/-}$ eliminan VACV de bazos y ovarios *in vivo* tan eficientemente como los ratones C57BL/6.

5. La vacunación de ratones TAP1$^{-/-}$y C57BL/6 con epítopos TAP-independientes de VACV mejora el efecto antiviral de los linfocitos T CD8$^{+}$.

6. Los epítopos TAP-independientes y sus precursores extendidos en amino son significativamente más hidrofóbicos que sus contrapartes TAP-dependientes.

## 8. BIBLIOGRAPHY.


Abele,R., Tampe,R., 1999. Function of the transport complex TAP in cellular immune recognition. Biochim. Biophys. Acta 1461**,** 405-419.

Abele,R., Tampé,R., 2004. The ABCs of immunology: structure and function of TAP, the transporter associated with antigen processing. Physiology. 19**,** 216-224.

Alcami,A., Smith,G.L., 1995. Vaccinia, cowpox, and camelpox viruses encode soluble gamma interferon receptors with novel broad species specificity. J. Virol. 69**,** 4633-4639.

Allikmets,R., Raskind,W.H., Hutchinson,A., Schueck,N.D., Dean,M., Koeller,D.M., 1999. Mutation of a putative mitochondrial iron transporter gene (ABC7) in X-linked sideroblastic anemia and ataxia (XLSA/A). Hum. Mol. Genet. 8**,** 743-749.

Alzhanova,D., Edwards,D.M., Hammarlund,E., Scholz,I.G., Horst,D., Wagner,M.J., Upton,C., Wiertz,E.J., Slifka,M.K., Fruh,K., 2009. Cowpox virus inhibits the transporter associated with antigen processing to evade T cell recognition. Cell Host. Microbe 6**,** 433-445.

Amanna,I.J., Slifka,M.K., Crotty,S., 2006. Immunity and immunological memory following smallpox vaccination. Immunol. Rev. 211**,** 320-337.

Anderson,K., Cresswell,P., Gammon,M., Hermes,J., Williamson,A., Zweerink,H., 1991. Endogenously synthesized peptide with an endoplasmic reticulum signal sequence sensitizes antigen processing mutant cells to class I-restricted cell-mediated lysis. J. Exp. Med. 174**,** 489-492.

Androlewicz,M.J., Cresswell,P., 1994. Human transporters associated with antigen processing possess a promiscuous peptide-binding site. Immunity 1**,** 7-14.

Annilo,T., Chen,Z.Q., Shulenin,S., Dean,M., 2003. Evolutionary analysis of a cluster of ATP-binding cassette (ABC) genes. Mamm. Genome 14**,** 7-20.

Antón,L.C., Snyder,H.L., Bennink,J.R., Vinitsky,A., Orlowski,M., Porgador,A., Yewdell,J.W., 1998. Dissociation of proteasomal degradation of biosynthesized viral proteins from generation of MHC class I-associated antigenic peptides. J. Immunol. 160**,** 4859-4868.

Bacik,I., Cox,J.H., Anderson,R., Yewdell,J.W., Bennink,J.R., 1994. TAP (transporter associated with antigen processing)-independent presentation of endogenously synthesized peptides is enhanced by endoplasmic reticulum insertion sequences located at the amino- but not carboxyl-terminus of the peptide. J. Immunol. 152**,** 381-387.

Baldick,C.J., Jr., Moss,B., 1993. Characterization and temporal regulation of mRNAs encoded by vaccinia virus intermediate-stage genes. J. Virol. 67**,** 3515-3527.

Bangert,I., Tumulka,F., Abele,R., 2011. The lysosomal polypeptide transporter TAPL: more than a housekeeping factor? Biol. Chem. 392**,** 61-66.

Baxby,D., 1977. The origins of vaccinia virus. J. Infect. Dis. 136**,** 453-455.

Becker,T., Volchuk,A., Rothman,J.E., 2005. Differential use of endoplasmic reticulum membrane for phagocytosis in J774 macrophages. Proc. Natl. Acad. Sci. U. S. A 102**,** 4022-4026.

Bell,M.J., Abbott,R.J., Croft,N.P., Hislop,A.D., Burrows,S.R., 2009. An HLA-A2-restricted T-cell epitope mapped to the BNLF2a immune evasion protein of Epstein-Barr virus that inhibits TAP. J Virol 83**,** 2783-2788.

Bengali,Z., Satheshkumar,P.S., Moss,B., 2012. Orthopoxvirus species and strain differences in cell entry. Virology 433**,** 506-512.

Bengali,Z., Satheshkumar,P.S., Yang,Z., Weisberg,A.S., Paran,N., Moss,B., 2011. Drosophila S2 cells are non-permissive for vaccinia virus DNA replication following entry via low pH-dependent endocytosis and early transcription. PLoS. ONE. 6**,** e17248.

Bengali,Z., Townsley,A.C., Moss,B., 2009. Vaccinia virus strain differences in cell attachment and entry. Virology 389**,** 132-140.

Beninga,J., Rock,K.L., Goldberg,A.L., 1998. Interferon-$\gamma$ can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. J. Biol. Chem. 273**,** 18734-18742.

Bidgood,S.R., Mercer,J., 2015. Cloak and Dagger: Alternative Immune Evasion and Modulation Strategies of Poxviruses. Viruses. 7**,** 4800-4825.

Bland,F.A., Lemberg,M.K., McMichael,A.J., Martoglio,B., Braud,V.M., 2003. Requirement of the proteasome for the trimming of signal peptide-derived epitopes presented by the nonclassical major histocompatibility complex class I molecule HLA-E. J. Biol. Chem. 278**,** 33747-33752.

Bomberger,J.M., Ely,K.H., Bangia,N., Ye,S., Green,K.A., Green,W.R., Enelow,R.I., Stanton,B.A., 2014. Pseudomonas aeruginosa Cif protein enhances the ubiquitination and proteasomal degradation of the transporter associated with antigen processing (TAP) and reduces major histocompatibility complex (MHC) class I antigen presentation. J. Biol. Chem. 289**,** 152-162.

Boname,J.M., May,J.S., Stevenson,P.G., 2005. The murine gamma-herpesvirus-68 MK3 protein causes TAP degradation independent of MHC class I heavy chain degradation. Eur. J. Immunol. 35**,** 171-179.

Brahmer,J.R., Drake,C.G., Wollner,I., Powderly,J.D., Picus,J., Sharfman,W.H., Stankevich,E., Pons,A., Salay,T.M., McMiller,T.L., Gilson,M.M., Wang,C., Selby,M., Taube,J.M., Anders,R., Chen,L., Korman,A.J., Pardoll,D.M., Lowy,I., Topalian,S.L., 2010. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. J. Clin. Oncol. 28**,** 3167-3175.

Broder,C.C., Kennedy,P.E., Michaels,F., Berger,E.A., 1994. Expression of foreign genes in cultured human primary macrophages using recombinant vaccinia virus vectors. Gene 142**,** 167-174.

Brutkiewicz,R.R., Klaus,S.J., Welsh,R.M., 1992. Window of vulnerability of vaccinia virus-infected cells to natural killer (NK) cell-mediated cytolysis correlates with enhanced NK cell triggering and is concomitant with a decrease in H-2 class I antigen expression. Nat. Immun. 11**,** 203-214.

Burrows,J.M., Bell,M.J., Brennan,R., Miles,J.J., Khanna,R., Burrows,S.R., 2007. Preferential binding of unusually long peptides to MHC class I and its influence on the selection of target peptides for T cell recognition. Mol. Immunol.

Byrd,D., Shepherd,N., Lan,J., Hu,N., Amet,T., Yang,K., Desai,M., Yu,Q., 2014. Primary human macrophages serve as vehicles for vaccinia virus replication and dissemination. J. Virol. 88**,** 6819-6831.

Cebrian,I., Visentin,G., Blanchard,N., Jouve,M., Bobard,A., Moita,C., Enninga,J., Moita,L.F., Amigorena,S., Savina,A., 2011. Sec22b regulates phagosomal maturation and antigen crosspresentation by dendritic cells. Cell 147**,** 1355-1368.

Chang,S.C., Momburg,F., Bhutani,N., Goldberg,A.L., 2005. The ER aminopeptidase, ERAP1, trims precursors to lengths of MHC class I peptides by a "molecular ruler" mechanism. Proc. Natl. Acad. Sci. U. S. A. 102**,** 17107-17112.

Chen,W., Antón,L.C., Bennink,J.R., Yewdell,J.W., 2000. Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses. Immunity 12**,** 83-93.

Childs,S., Ling,V., 1994. The MDR superfamily of genes and its biological implications. Important Adv. Oncol. 21-36.

Chisholm,S.E., Reyburn,H.T., 2006. Recognition of vaccinia virus-infected cells by human natural killer cells depends on natural cytotoxicity receptors. J. Virol. 80**,** 2225-2233.

Chung,C.S., Chen,C.H., Ho,M.Y., Huang,C.Y., Liao,C.L., Chang,W., 2006. Vaccinia virus proteome: identification of proteins in vaccinia virus intracellular mature virion particles. J. Virol. 80**,** 2127-2140.

Collins,E.J., Garboczi,D.N., Wiley,D.C., 1994. Three-dimensional structure of a peptide extending from one end of a class I MHC binding site. Nature 371**,** 626-629.

Croft,N.P., de Verteuil,D.A., Smith,S.A., Wong,Y.C., Schittenhelm,R.B., Tscharke,D.C., Purcell,A.W., 2015. Simultaneous Quantification of Viral Antigen Expression Kinetics Using Data-Independent (DIA) Mass Spectrometry. Mol. Cell Proteomics. 14**,** 1361-1372.

Croft,N.P., Shannon-Lowe,C., Bell,A.I., Horst,D., Kremmer,E., Ressing,M.E., Wiertz,E.J., Middeldorp,J.M., Rowe,M., Rickinson,A.B., Hislop,A.D., 2009. Stage-specific inhibition of MHC class I presentation by the Epstein-Barr virus BNLF2a protein during virus lytic cycle. PLoS. Pathog. 5**,** e1000490.

Croft,N.P., Smith,S.A., Wong,Y.C., Tan,C.T., Dudek,N.L., Flesch,I.E., Lin,L.C., Tscharke,D.C., Purcell,A.W., 2013. Kinetics of antigen expression and epitope presentation during virus infection. PLoS. Pathog. 9**,** e1003129.

Csere,P., Lill,R., Kispal,G., 1998. Identification of a human mitochondrial ABC transporter, the functional orthologue of yeast Atm1p. FEBS Lett. 441**,** 266-270.

Davison,A.J., Moss,B., 1989. Structure of vaccinia virus early promoters. J. Mol. Biol. 210**,** 749-769.

de Castro,I.F., Volonte,L., Risco,C., 2013. Virus factories: biogenesis and structural design. Cell Microbiol. 15**,** 24-34.

Del Val,M., Iborra,S., Ramos,M., Lázaro,S., 2011. Generation of MHC class I ligands in the secretory and vesicular pathways. Cell. Mol. Life Sci. 68**,** 1543-1552.

Del Val,M., Lazaro,S., Ramos,M., Antón,L.C., 2013. Are membrane proteins favored over cytosolic proteins in TAP-independent processing pathways? Mol. Immunol. 55**,** 117-119.

Del Val,M., López,D., 2002. Multiple proteases process viral antigens for presentation by MHC class I molecules to $CD8^+$ T lymphocytes. Mol. Immunol. 39**,** 235-247.

Demirel,O., Bangert,I., Tampe,R., Abele,R., 2010. Tuning the cellular trafficking of the lysosomal peptide transporter TAPL by its N-terminal domain. Traffic. 11**,** 383-393.

Demirel,O., Waibler,Z., Kalinke,U., Grunebach,F., Appel,S., Brossart,P., Hasilik,A., Tampe,R., Abele,R., 2007. Identification of a lysosomal peptide transport system induced during dendritic cell development. J. Biol. Chem. 282**,** 37836-37843.

Dolan,B.P., Bennink,J.R., Yewdell,J.W., 2011. Translating DRiPs: progress in understanding viral and cellular sources of MHC class I peptide ligands. Cell Mol. Life Sci. 68**,** 1481-1489.

Donaldson,J.G., Williams,D.B., 2009. Intracellular assembly and trafficking of MHC class I molecules. Traffic. 10**,** 1745-1752.

Dull,T., Zufferey,R., Kelly,M., Mandel,R.J., Nguyen,M., Trono,D., Naldini,L., 1998. A third-generation lentivirus vector with a conditional packaging system. J. Virol. 72**,** 8463-8471.

Earl,P.L., Americo,J.L., Wyatt,L.S., Eller,L.A., Whitbeck,J.C., Cohen,G.H., Eisenberg,R.J., Hartmann,C.J., Jackson,D.L., Kulesh,D.A., Martinez,M.J., Miller,D.M., Mucker,E.M., Shamblin,J.D., Zwiers,S.H., Huggins,J.W., Jahrling,P.B., Moss,B., 2004. Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox. Nature 428**,** 182-185.

Edghill-Smith,Y., Golding,H., Manischewitz,J., King,L.R., Scott,D., Bray,M., Nalca,A., Hooper,J.W., Whitehouse,C.A., Schmitz,J.E., Reimann,K.A., Franchini,G., 2005. Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. Nat. Med. 11**,** 740-747.

Eisenlohr,L.C., Bacik,I., Bennink,J.R., Bernstein,K., Yewdell,J.W., 1992. Expression of a membrane protease enhances presentation of endogenous antigens to MHC class I-restricted T lymphocytes. Cell 71**,** 963-972.

Elliott,T., Willis,A., Cerundolo,V., Townsend,A., 1995. Processing of major histocompatibility class I-restricted antigens in the endoplasmic reticulum. J. Exp. Med. 181**,** 1481-1491.

Evnouchidou,I., Momburg,F., Papakyriakou,A., Chroni,A., Leondiadis,L., Chang,S.C., Goldberg,A.L., Stratikos,E., 2008. The internal sequence of the peptide-substrate determines its N-terminus trimming by ERAP1. PLoS. ONE. 3**,** e3658.

Falk,K., Rotzschke,O., Stevanovic,S., Jung,G., Rammensee,H.G., 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 351**,** 290-296.

Fenteany,G., Standaert,R.F., Lane,W.S., Choi,S., Corey,E.J., Schreiber,S.L., 1995. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science 268**,** 726-731.

Firat,E., Huai,J., Saveanu,L., Gaedicke,S., Aichele,P., Eichmann,K., van Endert,P., Niedermann,G., 2007a. Analysis of Direct and Cross-Presentation of Antigens in TPPII Knockout Mice1. J Immunol. 179**,** 8137-8145.

Firat,E., Saveanu,L., Aichele,P., Staeheli,P., Huai,J., Gaedicke,S., Nil,A., Besin,G., Kanzler,B., van,E.P., Niedermann,G., 2007b. The role of endoplasmic reticulum-associated

aminopeptidase 1 in immunity to infection and in cross-presentation. J. Immunol. 178**,** 2241-2248.

Frank,N.Y., Margaryan,A., Huang,Y., Schatton,T., Waaga-Gasser,A.M., Gasser,M., Sayegh,M.H., Sadee,W., Frank,M.H., 2005. ABCB5-mediated doxorubicin transport and chemoresistance in human malignant melanoma. Cancer Res. 65**,** 4320-4333.

Furukawa,H., Murata,S., Yabe,T., Shimbara,N., Keicho,N., Kashiwase,K., Watanabe,K., Ishikawa,Y., Akaza,T., Tadokoro,K., Tohma,S., Inoue,T., Tokunaga,K., Yamamoto,K., Tanaka,K., Juji,T., 1999. Splice acceptor site mutation of the transporter associated with antigen processing-1 gene in human bare lymphocyte syndrome. J. Clin. Invest 103**,** 755-758.

Gadola,S.D., Moins-Teisserenc,H.T., Trowsdale,J., Gross,W.L., Cerundolo,V., 2000. TAP deficiency syndrome. Clin. Exp. Immunol. 121**,** 173-178.

Garcia-Medel,N., Sanz,A., Barnea,E., Admon,A., Lopez de Castro,J.A., 2011. The origin of proteasome-inhibitor resistant HLA class I peptidomes: a study with HLA-A*68:01. Mol. Cell Proteomics.

Garten,W., Hallenberger,S., Ortmann,D., Schafer,W., Vey,M., Angliker,H., Shaw,E., Klenk,H.D., 1994. Processing of viral glycoproteins by the subtilisin-like endoprotease furin and its inhibition by specific peptidylchloroalkylketones. Biochimie 76**,** 217-225.

Gasteiger,G., Kastenmuller,W., Ljapoci,R., Sutter,G., Drexler,I., 2007. Cross-priming of cytotoxic T cells dictates antigen requisites for modified vaccinia virus Ankara vector vaccines. J Virol 81**,** 11925-11936.

Geier,E., Pfeifer,G., Wilm,M., Lucchiari-Hartz,M., Baumeister,W., Eichmann,K., Niedermann,G., 1999. A giant protease with potential to substitute for some functions of the proteasome. Science 283**,** 978-981.

Gerloff,T., Stieger,B., Hagenbuch,B., Madon,J., Landmann,L., Roth,J., Hofmann,A.F., Meier,P.J., 1998. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J. Biol. Chem. 273**,** 10046-10050.

Gil-Torregrosa,B.C., Castaño,A.R., Del Val,M., 1998. Major histocompatibility complex class I viral antigen processing in the secretory pathway defined by the *trans*-Golgi network protease furin. J. Exp. Med. 188**,** 1105-1116.

Gil-Torregrosa,B.C., Castaño,A.R., López,D., Del Val,M., 2000. Generation of MHC class I peptide antigens by protein processing in the secretory route by furin. Traffic 1**,** 641-651.

Glas,R., Bogyo,M., Mcmaster,J.S., Gaczynska,M., Ploegh,H.L., 1998. A proteolytic system that compensates for loss of proteasome function. Nature 392**,** 618-622.

Goldenberg,M.M., 1999. Trastuzumab, a recombinant DNA-derived humanized monoclonal antibody, a novel agent for the treatment of metastatic breast cancer. Clin. Ther. 21**,** 309-318.

Gonzalez-Villalobos,R.A., Shen,X.Z., Bernstein,E.A., Janjulia,T., Taylor,B., Giani,J.F., Blackwell,W.L., Shah,K.H., Shi,P.D., Fuchs,S., Bernstein,K.E., 2013. Rediscovering ACE: novel insights into the many roles of the angiotensin-converting enzyme. J. Mol. Med. (Berl) 91**,** 1143-1154.

Gordon,D.E., Bond,L.M., Sahlender,D.A., Peden,A.A., 2010. A targeted siRNA screen to identify SNAREs required for constitutive secretion in mammalian cells. Traffic. 11**,** 1191-1204.

Gottesman,M.M., Fojo,T., Bates,S.E., 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat. Rev. Cancer 2**,** 48-58.

Groll,M., Ditzel,L., Lowe,J., Stock,D., Bochtler,M., Bartunik,H.D., Huber,R., 1997. Structure of 20S proteasome from yeast at 2.4 A resolution. Nature 386**,** 463-471.

Gromme,M., Neefjes,J., 2002. Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways. Mol. Immunol. 39**,** 181-202.

Gueguen,M., Biddison,W.E., Long,E.O., 1994. T cell recognition of an HLA-A2-restricted epitope derived from a cleaved signal sequence. J Exp. Med 180**,** 1989-1994.

Halle,S., Keyser,K.A., Stahl,F.R., Busche,A., Marquardt,A., Zheng,X., Galla,M., Heissmeyer,V., Heller,K., Boelter,J., Wagner,K., Bischoff,Y., Martens,R., Braun,A., Werth,K., Uvarovskii,A., Kempf,H., Meyer-Hermann,M., Arens,R., Kremer,M., Sutter,G., Messerle,M., Forster,R., 2016. In Vivo Killing Capacity of Cytotoxic T Cells Is Limited and Involves Dynamic Interactions and T Cell Cooperativity. Immunity. 44**,** 233-245.

Hammer,G.E., Gonzalez,F., James,E., Nolla,H., Shastri,N., 2007. In the absence of aminopeptidase ERAAP, MHC class I molecules present many unstable and highly immunogenic peptides. Nat. Immunol. 8**,** 101-108.

Harrington,L.E., Most,R., Whitton,J.L., Ahmed,R., 2002. Recombinant vaccinia virus-induced T-cell immunity: quantitation of the response to the virus vector and the foreign epitope. J. Virol. 76**,** 3329-3337.

Hay,J.C., Chao,D.S., Kuo,C.S., Scheller,R.H., 1997. Protein interactions regulating vesicle transport between the endoplasmic reticulum and Golgi apparatus in mammalian cells. Cell 89**,** 149-158.

Hearn,A., York,I.A., Rock,K.L., 2009. The specificity of trimming of MHC class I-presented peptides in the endoplasmic reticulum. J Immunol. 183**,** 5526-5536.

Heath,W.R., Carbone,F.R., 2001. Cross-presentation, dendritic cells, tolerance and immunity. Annu. Rev. Immunol. 19**,** 47-64.

Henderson,R.A., Michel,H., Sakaguchi,K., Shabanowitz,J., Appella,E., Hunt,D.F., Engelhard,V.H., 1992. HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. Science 255**,** 1264-1266.

Hewitt,E.W., Gupta,S.S., Lehner,P.J., 2001. The human cytomegalovirus gene product US6 inhibits ATP binding by TAP. EMBO J. 20**,** 387-396.

Hickman,H.D., Reynoso,G.V., Ngudiankama,B.F., Rubin,E.J., Magadan,J.G., Cush,S.S., Gibbs,J., Molon,B., Bronte,V., Bennink,J.R., Yewdell,J.W., 2013. Anatomically restricted synergistic antiviral activities of innate and adaptive immune cells in the skin. Cell Host. Microbe 13**,** 155-168.

Higgins,C.F., 2001. ABC transporters: physiology, structure and mechanism--an overview. Res. Microbiol. 152**,** 205-210.

Hiller,G., Weber,K., 1985. Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. J. Virol. 55**,** 651-659.

Hinz,A., Tampe,R., 2012. ABC transporters and immunity: mechanism of self-defense. Biochemistry 51**,** 4981-4989.

Hollinshead,M., Vanderplasschen,A., Smith,G.L., Vaux,D.J., 1999. Vaccinia virus intracellular mature virions contain only one lipid membrane. J. Virol. 73**,** 1503-1517.

Houot,R., Schultz,L.M., Marabelle,A., Kohrt,H., 2015. T-cell-based Immunotherapy: Adoptive Cell Transfer and Checkpoint Inhibition. Cancer Immunol. Res. 3**,** 1115-1122.

Huppa,J.B., Axmann,M., Mortelmaier,M.A., Lillemeier,B.F., Newell,E.W., Brameshuber,M., Klein,L.O., Schutz,G.J., Davis,M.M., 2010. TCR-peptide-MHC interactions in situ show accelerated kinetics and increased affinity. Nature 463**,** 963-967.

Husain,M., Weisberg,A.S., Moss,B., 2006. Existence of an operative pathway from the endoplasmic reticulum to the immature poxvirus membrane. Proc. Natl. Acad. Sci. U. S. A 103**,** 19506-19511.

Ichikawa,Y., Bayeva,M., Ghanefar,M., Potini,V., Sun,L., Mutharasan,R.K., Wu,R., Khechaduri,A., Jairaj,N.T., Ardehali,H., 2012. Disruption of ATP-binding cassette B8 in mice leads to cardiomyopathy through a decrease in mitochondrial iron export. Proc. Natl. Acad. Sci. U. S. A 109**,** 4152-4157.

Infantes,S., Samino,Y., Lorente,E., Jiménez,M., García,R., Del Val,M., López,D., 2010. H-2Ld class I molecule protects an HIV N-extended epitope from in vitro trimming by endoplasmic reticulum aminopeptidase associated with antigen processing. J Immunol. 184**,** 3351-3355.

Jacobs,N., Chen,R.A., Gubser,C., Najarro,P., Smith,G.L., 2006. Intradermal immune response after infection with Vaccinia virus. J. Gen. Virol. 87**,** 1157-1161.

Johnson,N., Vilardi,F., Lang,S., Leznicki,P., Zimmermann,R., High,S., 2012. TRC40 can deliver short secretory proteins to the Sec61 translocon. J Cell Sci 125**,** 3612-3620.

Johnstone,C., de León,P., Medina,F., Melero,J.A., García-Barreno,B., Del Val,M., 2004. Shifting immunodominance pattern of two cytotoxic T-lymphocyte epitopes in the F glycoprotein of the Long strain of respiratory syncytial virus. J. Gen. Virol. 85**,** 3229-3238.

Jones,J.F., 1982. Interactions between human neutrophils and vaccinia virus: induction of oxidative metabolism and virus inactivation. Pediatr. Res. 16**,** 525-529.

Kanaseki,T., Blanchard,N., Hammer,G.E., Gonzalez,F., Shastri,N., 2006. ERAAP synergizes with MHC class I molecules to make the final cut in the antigenic peptide precursors in the endoplasmic reticulum. Immunity. 25**,** 795-806.

Karttunen,J., Sanderson,S., Shastri,N., 1992. Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. Proc. Natl. Acad. Sci. U. S. A 89**,** 6020-6024.

Keck,J.G., Baldick,C.J., Jr., Moss,B., 1990. Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late trans-activator genes. Cell 61**,** 801-809.

Kessler,B., Hong,X., Petrovic,J., Borodovsky,A., Dantuma,N.P., Bogyo,M., Overkleeft,H.S., Ploegh,H., Glas,R., 2003. Pathways accessory to proteasomal proteolysis are less efficient in major histocompatibility complex class I antigen production. J. Biol. Chem. 278**,** 10013-10021.

Kessler,J.H., Khan,S., Seifert,U., Le Gall,S., Chow,K.M., Paschen,A., Bres-Vloemans,S.A., de Ru,A., van Montfoort,N., Franken,K.L., Benckhuijsen,W.E., Brooks,J.M., van Hall,T., Ray,K., Mulder,A., Doxiadis,I.I., van Swieten,P.F., Overkleeft,H.S., Prat,A., Tomkinson,B., Neefjes,J., Kloetzel,P.M., Rodgers,D.W., Hersh,L.B., Drijfhout,J.W., van Veelen,P.A., Ossendorp,F., Melief,C.J., 2011. Antigen processing by nardilysin and thimet oligopeptidase generates cytotoxic T cell epitopes. Nat. Immunol. 12**,** 45-53.

Khanna,R., Burrows,S.R., Moss,D.J., Silins,S.L., 1996. Peptide transporter (TAP-1 and TAP-2)-independent endongenous processing of Epstein-Barr virus (EBV) latent membrane protein 2A - implications for cytotoxic T-Lymphocyte control of EBV-associated malignancies. J. Virology 70**,** 5357-5362.

Kim,S., Lee,S., Shin,J., Kim,Y., Evnouchidou,I., Kim,D., Kim,Y.K., Kim,Y.E., Ahn,J.H., Riddell,S.R., Stratikos,E., Kim,V.N., Ahn,K., 2011. Human cytomegalovirus microRNA miR-US4-1 inhibits CD8(+) T cell responses by targeting the aminopeptidase ERAP1. Nat. Immunol. 12**,** 984-991.

Kisselev,A.F., Akopian,T.N., Woo,K.M., Goldberg,A.L., 1999. The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes - Implications for understanding the degradative mechanism and antigen presentation. J. Biol. Chem. 274**,** 3363-3371.

Kloetzel,P.M., Ossendorp,F., 2004. Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. Curr. Opin. Immunol. 16**,** 76-81.

Kobayashi,A., Kasano,M., Maeda,T., Hori,S., Motojima,K., Suzuki,M., Fujiwara,T., Takahashi,E., Yabe,T., Tanaka,K., Kasahara,M., Yamaguchi,Y., Maeda,M., 2000. A half-type ABC transporter TAPL is highly conserved between rodent and man, and the human gene is not responsive to interferon-gamma in contrast to TAP1 and TAP2. J Biochem. (Tokyo) 128**,** 711-718.

Koch,J., Guntrum,R., Heintke,S., Kyritsis,C., Tampe,R., 2004. Functional dissection of the transmembrane domains of the transporter associated with antigen processing (TAP). J. Biol. Chem. 279**,** 10142-10147.

Kochan,G., Krojer,T., Harvey,D., Fischer,R., Chen,L., Vollmar,M., von,D.F., Kavanagh,K.L., Brown,M.A., Bowness,P., Wordsworth,P., Kessler,B.M., Oppermann,U., 2011. Crystal structures of the endoplasmic reticulum aminopeptidase-1 (ERAP1) reveal the molecular basis for N-terminal peptide trimming. Proc. Natl. Acad. Sci. U. S. A 108**,** 7745-7750.

Koppers-Lalic,D., Reits,E.A., Ressing,M.E., Lipinska,A.D., Abele,R., Koch,J., Marcondes,R.M., Admiraal,P., van Leeuwen,D., Bienkowska-Szewczyk,K., Mettenleiter,T.C., Rijsewijk,F.A., Tampe,R., Neefjes,J., Wiertz,E.J., 2005. Varicelloviruses avoid T cell recognition by UL49.5-mediated inactivation of the transporter associated with antigen processing. Proc. Natl Acad. Sci U. S. A 102**,** 5144-5149.

Koppers-Lalic,D., Verweij,M.C., Lipinska,A.D., Wang,Y., Quinten,E., Reits,E.A., Koch,J., Loch,S., Marcondes,R.M., Daus,F., Bienkowska-Szewczyk,K., Osterrieder,N., Mettenleiter,T.C., Heemskerk,M.H., Tampe,R., Neefjes,J.J., Chowdhury,S.I., Ressing,M.E., Rijsewijk,F.A., Wiertz,E.J., 2008. Varicellovirus UL 49.5 proteins differentially affect the

function of the transporter associated with antigen processing, TAP. PLoS. Pathog. 4, e1000080.

Kurts,C., Robinson,B.W., Knolle,P.A., 2010. Cross-priming in health and disease. Nat. Rev. Immunol. 10, 403-414.

Lacaille,V.G., Androlewicz,M.J., 1998. HERPES SIMPLEX VIRUS INHIBITOR ICP47 DESTABILIZES THE TRANSPORTER ASSOCIATED WITH ANTIGEN PROCESSING (TAP) HETERODIMER. Journal of Biological Chemistry 273, 17386-17390.

Lamberson,H.V., Dock,N.L., 1992. Prevention of transfusion-transmitted cytomegalovirus infection. Transfusion 32, 196-198.

Lampen,M.H., Verweij,M.C., Querido,B., van der Burg,S.H., Wiertz,E.J., van Hall,T., 2010. CD8+ T cell responses against TAP-inhibited cells are readily detected in the human population. J Immunol. 185, 6508-6517.

Lautscham,G., Mayrhofer,S., Taylor,G., Haigh,T., Leese,A., Rickinson,A., Blake,N., 2001. Processing of a multiple membrane spanning Epstein-Barr virus protein for $CD8^+$ T cell recognition reveals a proteasome-dependent, transporter associated with antigen processing-independent pathway. J. Exp. Med. 194, 1053-1068.

Lautscham,G., Rickinson,A., Blake,N., 2003. TAP-independent antigen presentation on MHC class I molecules: lessons from Epstein-Barr virus. Microbes. Infect. 5, 291-299.

Lázaro,S., Gamarra,D., Del Val,M., 2015. Proteolytic enzymes involved in MHC class I antigen processing: A guerrilla army that partners with the proteasome. Mol. Immunol. 68, 72-76.

Lee,S.P., Thomas,W.A., Blake,N.W., Rickinson,A.B., 1996. Transporter (TAP)-independent processing of a multiple membrane- spanning protein, the Epstein-Barr virus latent membrane protein 2. European Journal of Immunology 26, 1875-1883.

Leone,P., Shin,E.C., Perosa,F., Vacca,A., Dammacco,F., Racanelli,V., 2013a. MHC class I antigen processing and presenting machinery: organization, function, and defects in tumor cells. J. Natl. Cancer Inst. 105, 1172-1187.

Leone,P., Shin,E.C., Perosa,F., Vacca,A., Dammacco,F., Racanelli,V., 2013b. MHC Class I Antigen Processing and Presenting Machinery: Organization, Function, and Defects in Tumor Cells. Journal of the National Cancer Institute.

Leonhardt,R.M., Fiegl,D., Rufer,E., Karger,A., Bettin,B., Knittler,M.R., 2010. Post-endoplasmic reticulum rescue of unstable MHC class I requires proprotein convertase PC7. J Immunol. 184, 2985-2998.

Lévy,F., Burri,L., Morel,S., Peitrequin,A.L., Lévy,N., Bachi,A., Hellman,U., Van den Eynde,B.J., Servis,C., 2002. The final N-terminal trimming of a subaminoterminal proline-containing HLA class I-restricted antigenic peptide in the cytosol is mediated by two peptidases. J. Immunol. 169, 4161-4171.

Liesa,M., Qiu,W., Shirihai,O.S., 2012. Mitochondrial ABC transporters function: the role of ABCB10 (ABC-me) as a novel player in cellular handling of reactive oxygen species. Biochim. Biophys. Acta 1823, 1945-1957.

Lin,L.C., Flesch,I.E., Tscharke,D.C., 2013. Immunodomination during peripheral vaccinia virus infection. PLoS. Pathog. 9, e1003329.

Liu,L., Cooper,T., Howley,P.M., Hayball,J.D., 2014. From crescent to mature virion: vaccinia virus assembly and maturation. Viruses. 6**,** 3787-3808.

Livak,K.J., Schmittgen,T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25**,** 402-408.

Ljunggren,H.G., Karre,K., 1985. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. J. Exp. Med. 162**,** 1745-1759.

Ljunggren,H.G., Ohlen,C., Hoglund,P., Franksson,L., Karre,K., 1991. The RMA-S lymphoma mutant; consequences of a peptide loading defect on immunological recognition and graft rejection. Int. J. Cancer Suppl 6**,** 38-44.

Looker,K.J., Magaret,A.S., May,M.T., Turner,K.M., Vickerman,P., Gottlieb,S.L., Newman,L.M., 2015a. Global and Regional Estimates of Prevalent and Incident Herpes Simplex Virus Type 1 Infections in 2012. PLoS. ONE. 10**,** e0140765.

Looker,K.J., Magaret,A.S., Turner,K.M., Vickerman,P., Gottlieb,S.L., Newman,L.M., 2015b. Global estimates of prevalent and incident herpes simplex virus type 2 infections in 2012. PLoS. ONE. 10**,** e114989.

López,D., Del Val,M., 1997. Selective involvement of proteasomes and cysteine proteases in MHC class I antigen presentation. J. Immunol. 159**,** 5769-5772.

López,D., Garcia-Calvo,M., Smith,G., Del Val,M., 2010. Caspases in virus-infected cells contribute to recognition by $CD8^+$ T lymphocytes. J. Immunol. 184**,** 5193-5199.

Maruri-Avidal,L., Weisberg,A.S., Moss,B., 2011. Vaccinia virus L2 protein associates with the endoplasmic reticulum near the growing edge of crescent precursors of immature virions and stabilizes a subset of viral membrane proteins. J. Virol. 85**,** 12431-12441.

Mayerhofer,P.U., Tampe,R., 2015. Antigen translocation machineries in adaptive immunity and viral immune evasion. J. Mol. Biol. 427**,** 1102-1118.

Medina,F., Ramos,M., Iborra,S., de León,P., Rodríguez-Castro,M., Del Val,M., 2009. Furin-processed antigens targeted to the secretory route elicit functional $TAP1^{-/-}$ $CD8^+$ T lymphocytes *in vivo*. J. Immunol. 183**,** 4639-4647.

Merzougui,N., Kratzer,R., Saveanu,L., van Endert,P., 2011. A proteasome-dependent, TAP-independent pathway for cross-presentation of phagocytosed antigen. EMBO Rep. 12**,** 1257-1264.

Mester,G., Hoffmann,V., Stevanovic,S., 2011. Insights into MHC class I antigen processing gained from large-scale analysis of class I ligands. Cell Mol. Life Sci. 68**,** 1521-1532.

Mickley,L., Jain,P., Miyake,K., Schriml,L.M., Rao,K., Fojo,T., Bates,S., Dean,M., 2001. An ATP-binding cassette gene (ABCG3) closely related to the multidrug transporter ABCG2 (MXR/ABCP) has an unusual ATP-binding domain. Mamm. Genome 12**,** 86-88.

Miller,B.H., Olson,S.L., Turek,F.W., Levine,J.E., Horton,T.H., Takahashi,J.S., 2004. Circadian clock mutation disrupts estrous cyclicity and maintenance of pregnancy. Curr. Biol. 14**,** 1367-1373.

Mitsuhashi,N., Miki,T., Senbongi,H., Yokoi,N., Yano,H., Miyazaki,M., Nakajima,N., Iwanaga,T., Yokoyama,Y., Shibata,T., Seino,S., 2000. MTABC3, a novel mitochondrial ATP-binding cassette protein involved in iron homeostasis. J. Biol. Chem. 275**,** 17536-17540.

Moffat,J., Grueneberg,D.A., Yang,X., Kim,S.Y., Kloepfer,A.M., Hinkle,G., Piqani,B., Eisenhaure,T.M., Luo,B., Grenier,J.K., Carpenter,A.E., Foo,S.Y., Stewart,S.A., Stockwell,B.R., Hacohen,N., Hahn,W.C., Lander,E.S., Sabatini,D.M., Root,D.E., 2006. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell 124**,** 1283-1298.

Moss,B., 2001 *Poxviridae*: The viruses and their replication. In: Knipe,D.M., Howley,P.M. (Eds.), Lippincott Williams & Wilkins, Philadelphia, PA., pp. 2849-2883.

Moss,B., 2006. Poxvirus entry and membrane fusion. Virology 344**,** 48-54.

Moss,B., 2015. Poxvirus membrane biogenesis. Virology 479-480**,** 619-626.

Mossman,K., Upton,C., Buller,R.M., McFadden,G., 1995. Species specificity of ectromelia virus and vaccinia virus interferon-gamma binding proteins. Virology 208**,** 762-769.

Mota,B.E., Gallardo-Romero,N., Trindade,G., Keckler,M.S., Karem,K., Carroll,D., Campos,M.A., Vieira,L.Q., da Fonseca,F.G., Ferreira,P.C., Bonjardim,C.A., Damon,I.K., Kroon,E.G., 2011. Adverse events post smallpox-vaccination: insights from tail scarification infection in mice with Vaccinia virus. PLoS. ONE. 6**,** e18924.

Moutaftsi,M., Peters,B., Pasquetto,V., Tscharke,D.C., Sidney,J., Bui,H.H., Grey,H., Sette,A., 2006. A consensus epitope prediction approach identifies the breadth of murine T $CD8^+$ cell responses to vaccinia virus. Nat. Biotechnol. 24**,** 817-819.

Moutaftsi,M., Salek-Ardakani,S., Croft,M., Peters,B., Sidney,J., Grey,H., Sette,A., 2009. Correlates of protection efficacy induced by vaccinia virus-specific CD8+ T-cell epitopes in the murine intranasal challenge model. Eur. J. Immunol. 39**,** 717-722.

Moutaftsi,M., Tscharke,D.C., Vaughan,K., Koelle,D.M., Stern,L., Calvo-Calle,M., Ennis,F., Terajima,M., Sutter,G., Crotty,S., Drexler,I., Franchini,G., Yewdell,J.W., Head,S.R., Blum,J., Peters,B., Sette,A., 2010. Uncovering the interplay between CD8, CD4 and antibody responses to complex pathogens. Future. Microbiol. 5**,** 221-239.

Nagarajan,N.A., Gonzalez,F., Shastri,N., 2012. Nonclassical MHC class Ib-restricted cytotoxic T cells monitor antigen processing in the endoplasmic reticulum. Nat. Immunol. 13**,** 579-586.

Natuk,R.J., Welsh,R.M., 1987. Accumulation and chemotaxis of natural killer/large granular lymphocytes at sites of virus replication. J. Immunol. 138**,** 877-883.

Neefjes,J.J., Momburg,F., Hämmerling,G.J., 1993. Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. Science 261**,** 769-771.

Ng,S.B., Khoury,J.D., 2009. Epstein-Barr virus in lymphoproliferative processes: an update for the diagnostic pathologist. Adv. Anat. Pathol. 16**,** 40-55.

Nguyen,T.T., Chang,S.C., Evnouchidou,I., York,I.A., Zikos,C., Rock,K.L., Goldberg,A.L., Stratikos,E., Stern,L.J., 2011. Structural basis for antigenic peptide precursor processing by the endoplasmic reticulum aminopeptidase ERAP1. Nat. Struct. Mol. Biol. 18**,** 604-613.

Nijenhuis,M., Hammerling,G.J., 1996. MULTIPLE REGIONS OF THE TRANSPORTER ASSOCIATED WITH ANTIGEN PROCESSING (TAP) CONTRIBUTE TO ITS PEPTIDE BINDING SITE. Journal of Immunology 157**,** 5467-5477.

Oliveira,C.C., Querido,B., Sluijter,M., de Groot,A.F., van der Zee,R., Rabelink,M.J., Hoeben,R.C., Ossendorp,F., van der Burg,S.H., van,H.T., 2013. New role of signal peptide peptidase to liberate C-terminal peptides for MHC class I presentation. J. Immunol. 191**,** 4020-4028.

Oliveira,C.C., Querido,B., Sluijter,M., Derbinski,J., van der Burg,S.H., van Hall,T., 2011. Peptide transporter TAP mediates between competing antigen sources generating distinct surface MHC class I peptide repertoires. Eur J Immunol. 41**,** 3114-3124.

Oliveira,C.C., Sluijter,M., Querido,B., Ossendorp,F., van der Burg,S.H., van,H.T., 2014. Dominant contribution of the proteasome and metalloproteinases to TAP-independent MHC-I peptide repertoire. Mol. Immunol. 62**,** 129-136.

Oliveira,C.C., van Veelen,P.A., Querido,B., de Ru,A., Sluijter,M., Laban,S., van der Burg,S.H., Offringa,R., van Hall,T., 2009. The nonpolymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects. J Exp. Med 207**,** 207-221.

Oseroff,C., Kos,F., Bui,H.H., Peters,B., Pasquetto,V., Glenn,J., Palmore,T., Sidney,J., Tscharke,D.C., Bennink,J.R., Southwood,S., Grey,H.M., Yewdell,J.W., Sette,A., 2005. HLA class I-restricted responses to vaccinia recognize a broad array of proteins mainly involved in virulence and viral gene regulation. Proc. Natl. Acad. Sci. U. S. A 102**,** 13980-13985.

Oseroff,C., Peters,B., Pasquetto,V., Moutaftsi,M., Sidney,J., Panchanathan,V., Tscharke,D.C., Maillere,B., Grey,H., Sette,A., 2008. Dissociation between epitope hierarchy and immunoprevalence in CD8 responses to vaccinia virus western reserve. J. Immunol. 180**,** 7193-7202.

Parmentier,N., Stroobant,V., Colau,D., de Diesbach,P., Morel,S., Chapiro,J., van Endert,P., Van den Eynde,B.J., 2010. Production of an antigenic peptide by insulin-degrading enzyme. Nat. Immunol. 11**,** 449-454.

Pasquetto,V., Bui,H.H., Giannino,R., Mirza,F., Sidney,J., Oseroff,C., Tscharke,D.C., Irvine,K., Bennink,J.R., Peters,B., Southwood,S., Cerundolo,V., Grey,H., Yewdell,J.W., Sette,A., 2005. HLA-A*0201, HLA-A*1101, and HLA-B*0702 transgenic mice recognize numerous poxvirus determinants from a wide variety of viral gene products. J. Immunol. 175**,** 5504-5515.

Powis,S.J., Young,L.L., Joly,E., Barker,P.J., Richardson,L., Brandt,R.P., Melief,C.J., Howard,J.C., Butcher,G.W., 1996. The rat *cim* effect: TAP allele-dependent changes in a class I MHC anchor motif and evidence against C-terminal trimming of peptides in the ER. Immunity 4**,** 159-165.

Priestman,M.A., Wang,Q., Jernigan,F.E., Chowdhury,R., Schmidt,M., Lawrence,D.S., 2014. Multicolor Monitoring of the Proteasome's Catalytic Signature. ACS Chem. Biol.

Princiotta,M.F., Finzi,D., Qian,S.B., Gibbs,J., Schuchmann,S., Buttgereit,F., Bennink,J.R., Yewdell,J.W., 2003. Quantitating protein synthesis, degradation, and endogenous antigen processing. Immunity 18**,** 343-354.

Purcell,A.W., Croft,N.P., Tscharke,D.C., 2016. Immunology by numbers: quantitation of antigen presentation completes the quantitative milieu of systems immunology! Curr. Opin. Immunol. 40**,** 88-95.

Raafat,N., Sadowski-Cron,C., Mengus,C., Heberer,M., Spagnoli,G.C., Zajac,P., 2011. Preventing vaccinia virus class-I epitopes presentation by HSV-ICP47 enhances the immunogenicity of a TAP-independent cancer vaccine epitope. Int. J. Cancer.

Rammensee,H.G., Bachmann,J., Emmerich,N.P.N., Bachor,O.A., Stevanovic,S., 1999. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 50**,** 213-219.

Reading,P.C., Smith,G.L., 2003. A kinetic analysis of immune mediators in the lungs of mice infected with vaccinia virus and comparison with intradermal infection. J. Gen. Virol. 84**,** 1973-1983.

Reid,P.A., Watts,C., 1990. Cycling of cell-surface MHC glycoproteins through primaquine-sensitive intracellular compartments. Nature 346**,** 655-657.

Reits,E., Griekspoor,A., Neijssen,J., Groothuis,T., Jalink,K., van Veelen,P., Janssen,H., Calafat,J., Drijfhout,J.W., Neefjes,J., 2003. Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. Immunity 18**,** 97-108.

Reits,E., Neijssen,J., Herberts,C., Benckhuijsen,W., Janssen,L., Drijfhout,J.W., Neefjes,J., 2004. A major role for TPPII in trimming proteasomal degradation products for MHC class I antigen presentation. Immunity. 20**,** 495-506.

Ruetz,S., Gros,P., 1994. Phosphatidylcholine translocase: a physiological role for the mdr2 gene. Cell 77**,** 1071-1081.

Russell,C.D., Unger,S.A., Walton,M., Schwarze,J., 2017. The Human Immune Response to Respiratory Syncytial Virus Infection. Clin. Microbiol. Rev. 30**,** 481-502.

Samino,Y., López,D., Guil,S., Saveanu,L., van Endert,P.M., Del Val,M., 2006. A long N-terminal-extended nested set of abundant and antigenic major histocompatibility complex class I natural ligands from HIV envelope protein. J. Biol. Chem. 281**,** 6358-6365.

Sandberg,J.K., Chambers,B.J., van Kaer,L., Karre,K., Ljunggren,H.G., 1996. TAP1-deficient mice select a $CD8^+$ T cell repertoire that displays both diversity and peptide specificity. Eur. J Immunol. 26**,** 288-293.

Saric,T., Beninga,J., Graef,C.I., Akopian,T.N., Rock,K.L., Goldberg,A.L., 2001. Major histocompatibility complex class I-presented antigenic peptides are degraded in cytosolic extracts primarily by thimet oligopeptidase. J. Biol. Chem. 276**,** 36474-36481.

Saric,T., Chang,S.C., Hattori,A., York,I.A., Markant,S., Rock,K.L., Tsujimoto,M., Goldberg,A.L., 2002. An IFN-$\gamma$-induced aminopeptidase in the ER, ERAP1, trims precursors to MHC class I-presented peptides. Nat. Immunol. 3**,** 1169-1176.

Saric,T., Graef,C.I., Goldberg,A.L., 2004. Pathway for degradation of peptides generated by proteasomes: a key role for thimet oligopeptidase and other metallopeptidases. J. Biol. Chem. 279**,** 46723-46732.

Sasaki,S., Tamaki,Y., Nagata,K., Kobayashi,Y., 2011. Regulation of the estrous cycle by neutrophils via opioid peptides. J. Immunol. 187**,** 774-780.

Satheshkumar,P.S., Antón,L.C., Sanz,P., Moss,B., 2009. Inhibition of the ubiquitin-proteasome system prevents vaccinia virus DNA replication and expression of intermediate and late genes. J Virol 83**,** 2469-2479.

Saveanu,L., Carroll,O., Lindo,V., Del Val,M., López,D., Lepelletier,Y., Greer,F., Schomburg,L., Fruci,D., Niedermann,G., van Endert,P.M., 2005. Concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidase complexes in the endoplasmic reticulum. Nat. Immunol. 6**,** 689-697.

Schatz,M.M., Peters,B., Akkad,N., Ullrich,N., Martinez,A.N., Carroll,O., Bulik,S., Rammensee,H.G., van Endert,P., Holzhutter,H.G., Tenzer,S., Schild,H., 2008. Characterizing the N-terminal processing motif of MHC class I ligands. J Immunol. 180**,** 3210-3217.

Schinkel,A.H., Mayer,U., Wagenaar,E., Mol,C.A., van,D.L., Smit,J.J., van der Valk,M.A., Voordouw,A.C., Spits,H., van,T.O., Zijlmans,J.M., Fibbe,W.E., Borst,P., 1997. Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. Proc. Natl. Acad. Sci. U. S. A 94**,** 4028-4033.

Schinkel,A.H., Smit,J.J., van,T.O., Beijnen,J.H., Wagenaar,E., van,D.L., Mol,C.A., van der Valk,M.A., Robanus-Maandag,E.C., te Riele,H.P., ., 1994. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell 77**,** 491-502.

Schlosser,E., Otero,C., Wuensch,C., Kessler,B., Edelmann,M., Brunisholz,R., Drexler,I., Legler,D.F., Groettrup,M., 2007. A novel cytosolic class I antigen-processing pathway for endoplasmic-reticulum-targeted proteins. EMBO Rep. 8**,** 945-951.

Schramm,B., Locker,J.K., 2005. Cytoplasmic organization of POXvirus DNA replication. Traffic. 6**,** 839-846.

Schumacher,T.N., Schreiber,R.D., 2015. Neoantigens in cancer immunotherapy. Science 348**,** 69-74.

Serwold,T., Gaw,S., Shastri,N., 2001. ER aminopeptidases generate a unique pool of peptides for MHC class I molecules. Nat. Immunol. 2**,** 644-651.

Serwold,T., González,F., Kim,J., Jacob,R., Shastri,N., 2002. ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. Nature 419**,** 480-483.

Sette,A., Grey,H., Oseroff,C., Peters,B., Moutaftsi,M., Crotty,S., Assarsson,E., Greenbaum,J., Kim,Y., Kolla,R., Tscharke,D., Koelle,D., Johnson,R.P., Blum,J., Head,S., Sidney,J., 2009. Definition of epitopes and antigens recognized by vaccinia specific immune responses: their conservation in variola virus sequences, and use as a model system to study complex pathogens. Vaccine 27 Suppl 6**,** G21-G26.

Sette,A., Moutaftsi,M., Moyron-Quiroz,J., McCausland,M.M., Davies,D.H., Johnston,R.J., Peters,B., Rafii-El-Idrissi,B.M., Hoffmann,J., Su,H.P., Singh,K., Garboczi,D.N., Head,S., Grey,H., Felgner,P.L., Crotty,S., 2008. Selective CD4+ T cell help for antibody responses to a large viral pathogen: deterministic linkage of specificities. Immunity. 28**,** 847-858.

Shao,S., Hegde,R.S., 2011. A calmodulin-dependent translocation pathway for small secretory proteins. Cell 147**,** 1576-1588.

Sharma,R.C., Inoue,S., Roitelman,J., Schimke,R.T., Simoni,R.D., 1992. Peptide transport by the multidrug resistance pump. J. Biol. Chem. 267**,** 5731-5734.

Sharom,F.J., 2011. The P-glycoprotein multidrug transporter. Essays Biochem. 50**,** 161-178.

Shastri,N., Cardinaud,S., Schwab,S.R., Serwold,T., Kunisawa,J., 2005. All the peptides that fit: the beginning, the middle, and the end of the MHC class I antigen-processing pathway. Immunol. Rev. 207**,** 31-41.

Shen,X.Z., Billet,S., Lin,C., Okwan-Duodu,D., Chen,X., Lukacher,A.E., Bernstein,K.E., 2011. The carboxypeptidase ACE shapes the MHC class I peptide repertoire. Nat. Immunol. 12**,** 1078-1085.

Shepard,A.R., Jacobson,N., Clark,A.F., 2005. Importance of quantitative PCR primer location for short interfering RNA efficacy determination. Anal. Biochem. 344**,** 287-288.

Shepherd,J.C., Schumacher,T.N., Ashton-Rickardt,P.G., Imaeda,S., Ploegh,H.L., Janeway,C.A.Jr., Tonegawa,S., 1993. TAP1-dependent peptide translocation *in vitro* is ATP dependent and peptide selective. Cell 74**,** 577-584.

Sigal,L.J., Rock,K.L., 2000. Bone marrow-derived antigen-presenting cells are required for the generation of cytotoxic T lymphocyte responses to viruses and use transporter associated with antigen presentation (TAP)-dependent and -independent pathways of antigen presentation. J. Exp. Med. 192**,** 1143-1150.

Sijts,E.J., Kloetzel,P.M., 2011. The role of the proteasome in the generation of MHC class I ligands and immune responses. Cell Mol. Life Sci. 68**,** 1491-1502.

Snyder,H.L., Bacík,I., Yewdell,J.W., Behrens,T.W., Bennink,J.R., 1998. Promiscuous liberation of MHC-class I-binding peptides from the C termini of membrane and soluble proteins in the secretory pathway. Eur. J. Immunol. 28**,** 1339-1346.

Snyder,H.L., Yewdell,J.W., Bennink,J.R., 1994. Trimming of antigenic peptides in an early secretory compartment. J. Exp. Med. 180**,** 2389-2394.

Stoltze,L., Schirle,M., Schwarz,G., Schröter,C., Thompson,M.W., Hersh,L.B., Kalbacher,H., Stevanovic,S., Rammensee,H.G., Schild,H., 2000. Two new proteases in the MHC class I processing pathway. Nat. Immunol. 1**,** 413-418.

Suri,A., Walters,J.J., Levisetti,M.G., Gross,M.L., Unanue,E.R., 2006. Identification of naturally processed peptides bound to the class I MHC molecule H-2K$^{d}$ of normal and TAP-deficient cells. Eur. J. Immunol. 36**,** 544-557.

Sweet,R.M., Eisenberg,D., 1983. Correlation of sequence hydrophobicities measures similarity in three-dimensional protein structure. J. Mol. Biol. 171**,** 479-488.

Szajner,P., Weisberg,A.S., Lebowitz,J., Heuser,J., Moss,B., 2005. External scaffold of spherical immature poxvirus particles is made of protein trimers, forming a honeycomb lattice. J. Cell Biol. 170**,** 971-981.

Tammur,J., Prades,C., Arnould,I., Rzhetsky,A., Hutchinson,A., Adachi,M., Schuetz,J.D., Swoboda,K.J., Ptacek,L.J., Rosier,M., Dean,M., Allikmets,R., 2001. Two new genes from the human ATP-binding cassette transporter superfamily, ABCC11 and ABCC12, tandemly duplicated on chromosome 16q12. Gene 273**,** 89-96.

Terajima,M., Cruz,J., Leporati,A.M., Demkowicz,W.E., Jr., Kennedy,J.S., Ennis,F.A., 2006. Identification of vaccinia CD8+ T-cell epitopes conserved among vaccinia and variola viruses restricted by common MHC class I molecules, HLA-A2 or HLA-B7. Hum. Immunol. 67**,** 512-520.

Theodoulou,F.L., Kerr,I.D., 2015. ABC transporter research: going strong 40 years on. Biochem. Soc. Trans. 43**,** 1033-1040.

Tiwari,N., Garbi,N., Reinheckel,T., Moldenhauer,G., Hammerling,G.J., Momburg,F., 2007. A transporter associated with antigen-processing independent vacuolar pathway for the MHC

class I-mediated presentation of endogenous transmembrane proteins. J. Immunol. 178**,** 7932-7942.

Tolonen,N., Doglio,L., Schleich,S., Krijnse,L.J., 2001. Vaccinia virus DNA replication occurs in endoplasmic reticulum-enclosed cytoplasmic mini-nuclei. Mol. Biol. Cell 12**,** 2031-2046.

Tomkinson,B., 1999. Tripeptidyl peptidases: enzymes that count. Trends Biochem. Sci. 24**,** 355-359.

Tooze,J., Hollinshead,M., Reis,B., Radsak,K., Kern,H., 1993. Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. Eur. J. Cell Biol. 60**,** 163-178.

Topalian,S.L., Hodi,F.S., Brahmer,J.R., Gettinger,S.N., Smith,D.C., McDermott,D.F., Powderly,J.D., Carvajal,R.D., Sosman,J.A., Atkins,M.B., Leming,P.D., Spigel,D.R., Antonia,S.J., Horn,L., Drake,C.G., Pardoll,D.M., Chen,L., Sharfman,W.H., Anders,R.A., Taube,J.M., McMiller,T.L., Xu,H., Korman,A.J., Jure-Kunkel,M., Agrawal,S., Mcdonald,D., Kollia,G.D., Gupta,A., Wigginton,J.M., Sznol,M., 2012. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N. Engl. J. Med. 366**,** 2443-2454.

Towne,C.F., York,I.A., Neijssen,J., Karow,M.L., Murphy,A.J., Valenzuela,D.M., Yancopoulos,G.D., Neefjes,J.J., Rock,K.L., 2005. Leucine aminopeptidase is not essential for trimming peptides in the cytosol or generating epitopes for MHC class I antigen presentation. J. Immunol. 175**,** 6605-6614.

Towne,C.F., York,I.A., Neijssen,J., Karow,M.L., Murphy,A.J., Valenzuela,D.M., Yancopoulos,G.D., Neefjes,J.J., Rock,K.L., 2008. Puromycin-sensitive aminopeptidase limits MHC class I presentation in dendritic cells but does not affect CD8 T cell responses during viral infections. J Immunol. 180**,** 1704-1712.

Towne,C.F., York,I.A., Watkin,L.B., Lazo,J.S., Rock,K.L., 2007. Analysis of the role of bleomycin hydrolase in antigen presentation and the generation of CD8 T cell responses. J Immunol. 178**,** 6923-6930.

Tscharke,D.C., Croft,N.P., Doherty,P.C., La Gruta,N.L., 2015. Sizing up the key determinants of the CD8(+) T cell response. Nat. Rev. Immunol. 15**,** 705-716.

Tscharke,D.C., Karupiah,G., Zhou,J., Palmore,T., Irvine,K.R., Haeryfar,S.M., Williams,S., Sidney,J., Sette,A., Bennink,J.R., Yewdell,J.W., 2005. Identification of poxvirus $CD8^+$ T cell determinants to enable rational design and characterization of smallpox vaccines. J. Exp. Med. 201**,** 95-104.

Tscharke,D.C., Woo,W.P., Sakala,I.G., Sidney,J., Sette,A., Moss,D.J., Bennink,J.R., Karupiah,G., Yewdell,J.W., 2006. Poxvirus CD8+ T-cell determinants and cross-reactivity in BALB/c mice. J. Virol. 80**,** 6318-6323.

Tulman,E.R., Delhon,G., Afonso,C.L., Lu,Z., Zsak,L., Sandybaev,N.T., Kerembekova,U.Z., Zaitsev,V.L., Kutish,G.F., Rock,D.L., 2006. Genome of horsepox virus. J. Virol. 80**,** 9244-9258.

Turner,G.S., 1982. Jenner's smallpox vaccine-the riddle of vaccinia, virus and its origin by Derrick Baxby, Heineman Educational Books Ltd, 1981. pound8.50 (xiii + 214 pages) ISBN 0 435 54047 2. Immunol. Today 3**,** 26.

Turzynski,A., Mentlein,R., 1990. Prolyl aminopeptidase from rat brain and kidney. Action on peptides and identification as leucyl aminopeptidase. Eur. J. Biochem. 190**,** 509-515.

Uebel,S., Kraas,W., Kienle,S., Wiesmuller,K.H., Jung,G., Tampe,R., 1997. Recognition principle of the TAP transporter disclosed by combinatorial peptide libraries. Proc. Natl. Acad. Sci. U. S. A. 94**,** 8976-8981.

Uebel,S., Meyer,T.H., Kraas,W., Kienle,S., Jung,G., Wiesmuller,K.H., Tampe,R., 1995. Requirements for peptide binding to the human transporter associated with antigen processing revealed by peptide scans and complex peptide libraries. J. Biol. Chem. 270**,** 18512-18516.

van Endert P., 2016. Intracellular recycling and cross-presentation by MHC class I molecules. Immunol. Rev. 272**,** 80-96.

van Endert,P., 2008. Role of tripeptidyl peptidase II in MHC class I antigen processing - the end of controversies? Eur J Immunol. 38**,** 609-613.

van Endert,P.M., Riganelli,D., Greco,G., Fleischhauer,K., Sidney,J., Sette,A., Bach,J.F., 1995. The peptide-binding motif for the human transporter associated with antigen processing. J. Exp. Med. 182**,** 1883-1895.

van Endert,P.M., Tampé,R., Meyer,T.H., Tisch,R., Bach,J.F., McDevitt,H.O., 1994. A sequential model for peptide binding and transport by the transporters associated with antigen processing. Immunity. 1**,** 491-500.

van Hall,T., Wolpert,E.Z., van Veelen,P., Laban,S., van,d., V, Roseboom,M., Bres,S., Grufman,P., de Ru,A., Meiring,H., de Jong,A., Franken,K., Teixeira,A., Valentijn,R., Drijfhout,J.W., Koning,F., Camps,M., Ossendorp,F., Karre,K., Ljunggren,H.G., Melief,C.J., Offringa,R., 2006. Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. Nat. Med 12**,** 417-424.

van Helvoort,A., Smith,A.J., Sprong,H., Fritzsche,I., Schinkel,A.H., Borst,P., van Meer,G., 1996. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell 87**,** 507-517.

van Kaer,L., Ashton Rickardt,P.G., Ploegh,H.L., Tonegawa,S., 1992. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and $CD4^{-}8^{+}$ T cells. Cell 71**,** 1205-1214.

Verardi,P.H., Jones,L.A., Aziz,F.H., Ahmad,S., Yilma,T.D., 2001. Vaccinia virus vectors with an inactivated gamma interferon receptor homolog gene (B8R) are attenuated In vivo without a concomitant reduction in immunogenicity. J. Virol. 75**,** 11-18.

Wang,W., Bodles-Brakhop,A.M., Barger,S.W., 2016. A Role for P-Glycoprotein in Clearance of Alzheimer Amyloid beta -Peptide from the Brain. Curr. Alzheimer Res. 13**,** 615-620.

Wehmer,M., Sakata,E., 2016. Recent advances in the structural biology of the 26S proteasome. Int. J. Biochem. Cell Biol. 79**,** 437-442.

Wei,M.L., Cresswell,P., 1992. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. Nature 356**,** 443-446.

Weinzierl,A.O., Rudolf,D., Hillen,N., Tenzer,S., van Endert,P., Schild,H., Rammensee,H.G., Stevanovic,S., 2008. Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry. Eur J Immunol. 38**,** 1503-1510.

Wennier,S.T., Brinkmann,K., Steinhausser,C., Maylander,N., Mnich,C., Wielert,U., Dirmeier,U., Hausmann,J., Chaplin,P., Steigerwald,R., 2013. A novel naturally occurring

tandem promoter in modified vaccinia virus ankara drives very early gene expression and potent immune responses. PLoS. ONE. 8, e73511.

West,B.C., Eschete,M.L., Cox,M.E., King,J.W., 1987. Neutrophil uptake of vaccinia virus in vitro. J. Infect. Dis. 156, 597-606.

Wherry,E.J., Golovina,T.N., Morrison,S.E., Sinnathamby,G., McElhaugh,M.J., Shockey,D.C., Eisenlohr,L.C., 2006. Re-evaluating the generation of a "proteasome-independent" MHC class I-restricted CD8 T cell epitope. J. Immunol. 176, 2249-2261.

Wherry,E.J., Puorro,K.A., Porgador,A., Eisenlohr,L.C., 1999. The induction of virus-specific CTL as a function of increasing epitope expression: responses rise steadily until excessively high levels of epitope are attained. J. Immunol. 163, 3735-3745.

Wherry,E.J., Rajagopal,D., Eisenlohr,L.C., 2001. Use of vaccinia virus expression vectors to investigate antigen processing and presentation. Methods Mol. Biol. 156, 89-109.

Wiertz,E.J., Tortorella,D., Bogyo,M., Yu,J., Mothes,W., Jones,T.R., Rapoport,T.A., Ploegh,H.L., 1996. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction [see comments]. Nature 384, 432-438.

Wolpert,E.Z., Petersson,M., Chambers,B.J., Sandberg,J.K., Kiessling,R., Ljunggren,H.G., Karre,K., 1997. Generation of $CD8^+$ T cells specific for transporter associated with antigen processing deficient cells. Proc. Natl. Acad. Sci. USA 94, 11496-11501.

Wolters,J.C., Abele,R., Tampe,R., 2005. Selective and ATP-dependent translocation of peptides by the homodimeric ATP binding cassette transporter TAP-like (ABCB9). J Biol. Chem. 280, 23631-23636.

Xu,R., Johnson,A.J., Liggitt,D., Bevan,M.J., 2004. Cellular and humoral immunity against vaccinia virus infection of mice. J. Immunol. 172, 6265-6271.

Xu,R.H., Remakus,S., Ma,X., Roscoe,F., Sigal,L.J., 2010. Direct presentation is sufficient for an efficient anti-viral CD8+ T cell response. PLoS. Pathog. 6, e1000768.

Yamaguchi,Y., Kasano,M., Terada,T., Sato,R., Maeda,M., 1999. An ABC transporter homologous to TAP proteins. FEBS Lett. 457, 231-236.

Yamamoto,Y., Sakisaka,T., 2012. Molecular Machinery for Insertion of Tail-Anchored Membrane Proteins into the Endoplasmic Reticulum Membrane in Mammalian Cells. Mol Cell 48, 387-397.

Yan,J., Parekh,V.V., Mendez-Fernandez,Y., Olivares-Villagomez,D., Dragovic,S., Hill,T., Roopenian,D.C., Joyce,S., van Kaer,L., 2006. In vivo role of ER-associated peptidase activity in tailoring peptides for presentation by MHC class Ia and class Ib molecules. J. Exp. Med. 203, 647-659.

Yang,Z., Bruno,D.P., Martens,C.A., Porcella,S.F., Moss,B., 2010. Simultaneous high-resolution analysis of vaccinia virus and host cell transcriptomes by deep RNA sequencing. Proc. Natl. Acad. Sci. U. S. A 107, 11513-11518.

Yang,Z., Reynolds,S.E., Martens,C.A., Bruno,D.P., Porcella,S.F., Moss,B., 2011. Expression profiling of the intermediate and late stages of poxvirus replication. J. Virol. 85, 9899-9908.

Yewdell,J.W., Nicchitta,C.V., 2006. The DRiP hypothesis decennial: support, controversy, refinement and extension. Trends Immunol. 27, 368-373.

York,I.A., Bhutani,N., Zendzian,S., Goldberg,A.L., Rock,K.L., 2006. Tripeptidyl peptidase II is the major peptidase needed to trim long antigenic precursors, but is not required for most MHC class I antigen presentation. J. Immunol. 177**,** 1434-1443.

York,I.A., Chang,S.C., Saric,T., Keys,J.A., Favreau,J.M., Goldberg,A.L., Rock,K.L., 2002. The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8-9 residues. Nat. Immunol. 3**,** 1177-1184.

Yuen,T.J., Flesch,I.E., Hollett,N.A., Dobson,B.M., Russell,T.A., Fahrer,A.M., Tscharke,D.C., 2010. Analysis of A47, an immunoprevalent protein of vaccinia virus, leads to a reevaluation of the total antiviral CD8+ T cell response. J. Virol. 84**,** 10220-10229.

Zervoudi,E., Saridakis,E., Birtley,J.R., Seregin,S.S., Reeves,E., Kokkala,P., Aldhamen,Y.A., Amalfitano,A., Mavridis,I.M., James,E., Georgiadis,D., Stratikos,E., 2013. Rationally designed inhibitor targeting antigen-trimming aminopeptidases enhances antigen presentation and cytotoxic T-cell responses. Proc. Natl. Acad. Sci. U. S. A 110**,** 19890-19895.

Zhang,F., Zhang,W., Liu,L., Fisher,C.L., Hui,D., Childs,S., Dorovini-Zis,K., Ling,V., 2000. Characterization of ABCB9, an ATP binding cassette protein associated with lysosomes. J Biol. Chem. 275**,** 23287-23294.

Zhang,T., Wong,S.H., Tang,B.L., Xu,Y., Hong,W., 1999. Morphological and functional association of Sec22b/ERS-24 with the pre-Golgi intermediate compartment. Mol. Biol. Cell 10**,** 435-453.

Zhao,C., Tampe,R., Abele,R., 2006. TAP and TAP-like--brothers in arms? Naunyn Schmiedebergs Arch. Pharmacol. 372**,** 444-450.

Zhou,X., Glas,R., Momburg,F., Hammerling,G.J., Jondal,M., Ljunggren,H.G., 1993. TAP2-defective RMA-S cells present Sendai virus antigen to cytotoxic T lymphocytes. Eur. J Immunol. 23**,** 1796-1801.

Zhu,H., Fearnhead,H.O., Cohen,G.M., 1995. An ICE-like protease is a common mediator of apoptosis induced by diverse stimuli in human monocytic THP.1 cells. FEBS Lett. 374**,** 303-308.

Zolnerciks,J.K., Andress,E.J., Nicolaou,M., Linton,K.J., 2011. Structure of ABC transporters. Essays Biochem. 50**,** 43-61.